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STUDIES ON BASOPHILIA OF NUCLEIC ACIDS: THE METHYL GREEN STAINABILITY OF NUCLEIC ACIDS[1]

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A number of reports in the recent cytochemical literature have dealt with the use of the basic dye methyl green as a specific and quantitative stain of desoxyribonucleic acid (DNA). Pollister and Leuchtenberger (1949) first demonstrated by photometric methods the high degree of reproducibility of nuclear methyl green staining in mouse liver and discussed the impairment of stainability caused by pretreatment of slides with hot water. Kurnick (1950a) concluded from test tube experiments that methyl green combines specifically with highly polymerized DNA only. He used the stain for quantitative determinations of DNA in fixed histological material (1950b; Kurnick and Herskowitz, 1951) and to measure depolymerase activity of serum (1950c). Vercauteren (1950) and Devreux *et al.* (1951) also tested the dye-binding capacity of DNA solutions under various conditions and concur with Kurnick's opinion. The methyl green stainability of some types of degenerating chromatin was investigated by Leuchtenberger (1950), Klemperer *et al.* (1950) and Korson (1951), and the influence of x-radiation on stainability of nuclei was tested by Moses *et al.* (1951) and Harrington and Koza (1951).

Impairment of methyl green stainability as it was found to occur in some instances, and as it can be induced by treatment of slides with hot water or mild acid hydrolysis, would, according to Kurnick's view, be due to a depolymerization of the DNA. Pollister and Leuchtenberger (1949) have also used the term "depolymerization" to designate an unspecified change in the configuration of the DNA molecule without implying that depolymerization in the chemical sense (breakage of internucleotide linkages) was necessarily involved.

Although methyl green has long been regarded as an exclusive nuclear stain, there are some indications that it does not possess absolute specificity for DNA, since its ability to distinguish DNA from ribonucleic acid (RNA) depends on the type of fixation used: following fixation in acetic alcohol, staining is entirely restricted to nuclei in many types of tissues, while after fixation in formalin, specificity is often lost and cytoplasmic RNA also binds methyl green strongly. Furthermore, in some tissues RNA is methyl green stainable even after acetic alcohol fixation.

[1] The work reported here started when the author was a Public Health Research Fellow of the National Institutes of Health. At present the author receives support from the University of California Board of Research and from California Cancer Research Funds.

This is notably the case in Nissl substance (as observed by Koenig, and by Swift and Flax, personal communications); we have also found it to occur in the sperm cytoplasm of *Ascaris megalocephala* and in the serous cells of the submaxillary gland of the mouse. In accordance with the concept proposed by Kurnick, it seemed possible that certain types of RNA occur in a highly polymerized state and are therefore able to combine with methyl green. We recorded a hypothesis to that effect in a recent abstract (Alfert, 1951) when we observed that smears of RNA from tobacco mosaic virus, which was known to be highly polymerized, could be stained with methyl green. However, further experiments demonstrated that this hypothesis was inadequate and that other factors, which have not been previously described, strongly influence the methyl green stainability of nucleic acids.

Materials and Methods

The staining reaction of nucleic acids in several animal and one plant tissue was investigated. All tissues were fixed in Carnoy's acetic alcohol and paraffin sections of different materials were mounted together to be treated and stained under identical conditions. Several nucleic acid and nucleoprotein model systems [2] were also used; artificial fibers of nucleohistone prepared from calf thymus were fixed, embedded and sectioned like tissues, and drops of solutions were allowed to dry on slides, fixed in acetic alcohol or ethyl alcohol, and stained. The usefulness of the latter staining models is rather limited because variable amounts of the substrate are likely to be lost from the slides during the staining procedure. Only positive results are significant and constitute at best a qualitative test since a reliable comparison of relative staining intensities is not possible.

Methyl green, Natl. Aniline Div., C.I. No. 685, NG26 was purified by chloroform extraction and dissolved in the phenol-glycerin medium used by Pollister and Leuchtenberger (1949) in a concentration of 0.25%. The pH of this solution is 4.5. Materials were stained for 90 minutes at 37° C., rinsed in distilled water, blotted and differentiated for at least 18 hours in tertiary butyl alcohol. This method results in an excellent and, as shown by photometric measurements, very reproducible stain. For histological purposes we found this staining method superior to that proposed by Kurnick (1950b) since staining is more intense and even throughout the section. Both methods are of equal specificity when tested on the same objects.

The methyl green stainability of all substrates was compared to their general basophilia tested with the basic stain azure B. As described by Flax and Himes (1951), this stain permits visualization of DNA and RNA in different colors, blue and purple, respectively. Occasionally other basic stains were used in aqueous and buffered solutions, and the Feulgen reaction was applied (12 minutes hydrolysis in 1 N HCl at 60° C.).

Removability of the stainable substrates by hot trichloroacetic acid and by specific nucleases was used to insure the specificity of the staining reaction for nucleic acids.

A number of treatments which affect basophilia of nucleic acids were applied to fixed materials prior to staining:

[2] We are greatly indebted to Dr. C. A. Knight as well as to Dr. D. Mazia and members of their laboratories for supplying the nucleic acid and nucleoprotein extracts used in this investigation.

1. Immersion of slides into hot distilled water (90°–95° C.) for 30–60 minutes.
2. Hydrolysis in 1 N HCl at 60° C. for 12 minutes, and immersion in dilute acid (0.01 N HCl) and alkali (0.01 N NaOH) for 24 hours at room temperature.
3. Van Slyke reaction as applied by Ornstein and Flax (unpublished) to remove amino groups of proteins: 10 g. $NaNO_2$ in 45 cc. 50% ethyl alcohol and 15 cc. glacial acetic acid; slides treated for two hours at room temperature.
4. Acetylation of amino groups by acetic anhydride: slides blotted dry out of absolute alcohol and immersed for one hour at room temperature.

A comparison of the relative staining intensity of nuclei was made by means of photometric techniques described elsewhere (Pollister and Ris, 1947; Swift, 1950). The apparatus is similar to that described by Pollister and Moses (1949) and a Beckman B spectrophotometer was used as a light source. The Feulgen reagent was measured at 550 mμ, and methyl green at 630 mμ, slightly off the absorption peak of the dye combined with nucleic acids. Measured values are reported in arbitrary units of dye per nucleus. The tables give mean values and standard errors obtained in several series of measurements.

Experiments and Observations

1. Cytoplasmic basophilia and staining models

Plate 1 contains photomicrographs of four different tissues of a mouse, each stained with azure B and methyl green for comparison. The tissues in the first vertical column—liver, pancreas, submaxillary gland and spinal cord—were stained with azure and the presence of high concentrations of RNA can thereby be demonstrated in all of them. The staining picture of the same tissues with methyl green (second column) is quite different, however; in liver and pancreas the methyl green picture is completely equivalent to a Feulgen preparation of the same material, only nuclei stain and the cytoplasm remains unstained. In the submaxillary gland, however, the cytoplasmic RNA of serous cells combines with methyl green and the same is true for Nissl substance in the motor neurons of the spinal cord. Comparing the two photographs of motor neurons, it is evident that the strongly azurophilic nucleolus does not stain with methyl green. Figure 9 on Plate II is a methyl green preparation of a fertilized egg of Ascaris. In addition to the egg chromosomes and the small sperm nucleus, the whole body of the amoeboid sperm stains strongly with methyl green.

Thus there exist clear-cut differences among ribonucleic acids in different tissues with respect to their ability to bind methyl green. Using model systems of different nucleic acid and nucleoprotein solutions, we were unable to find qualitative differences in stainability. The following systems were investigated:

1. Calf thymus nucleohistone.
2. Calf thymus nucleohistone after irradiation with a dose of 100,000 r (Himes and Alfert, unpublished).
3. Nucleoprotein from sea urchin sperm.
4. 0.1% solution of DNA from chicken erythrocytes.
5. 0.1% solution of yeast RNA (Schwarz).
6. 0.1% solution of tobacco mosaic virus RNA.

7–10. Solutions 3–6 after heating for one hour in a bath of boiling water.

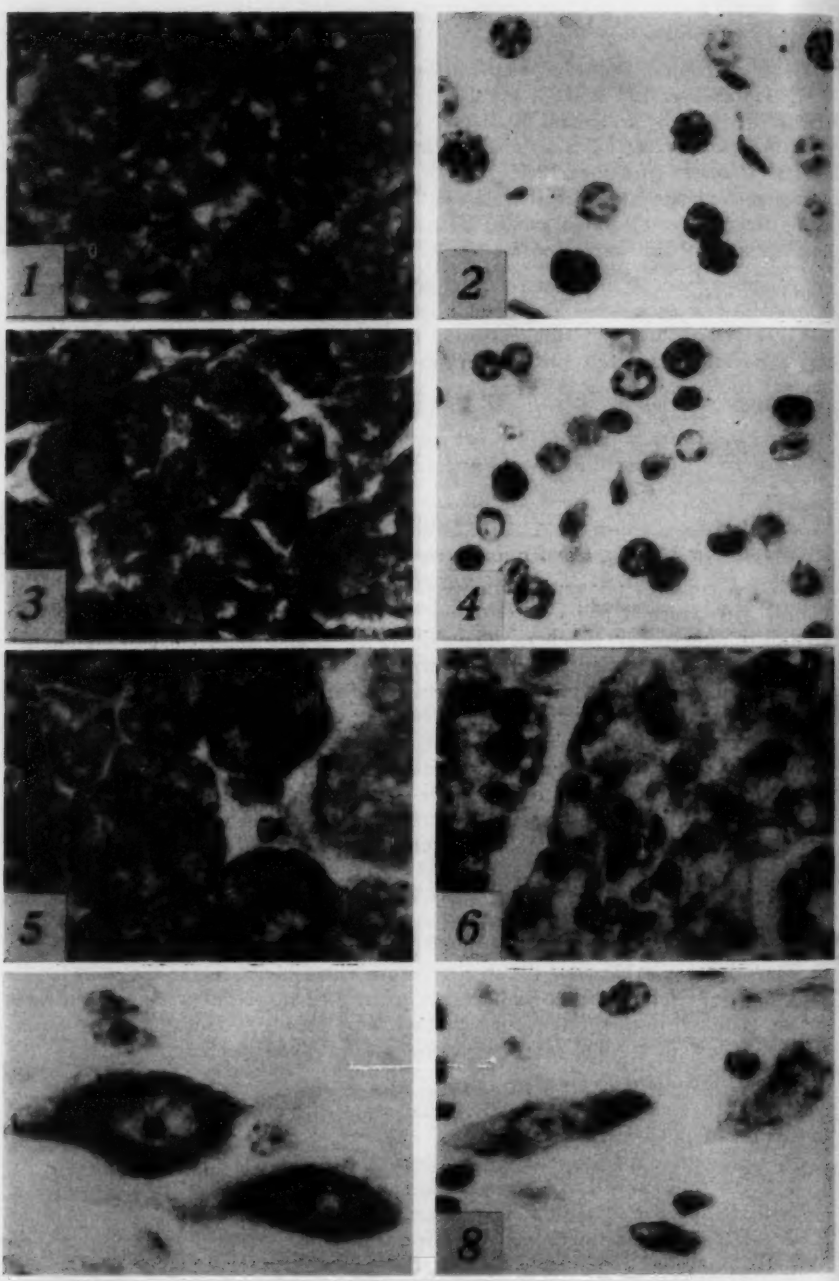

PLATE I. Mouse tissues fixed in Carnoy's acetic-alcohol; 10 μ sections, × 750.
(Photomicrography by Mr. Victor Duran.)

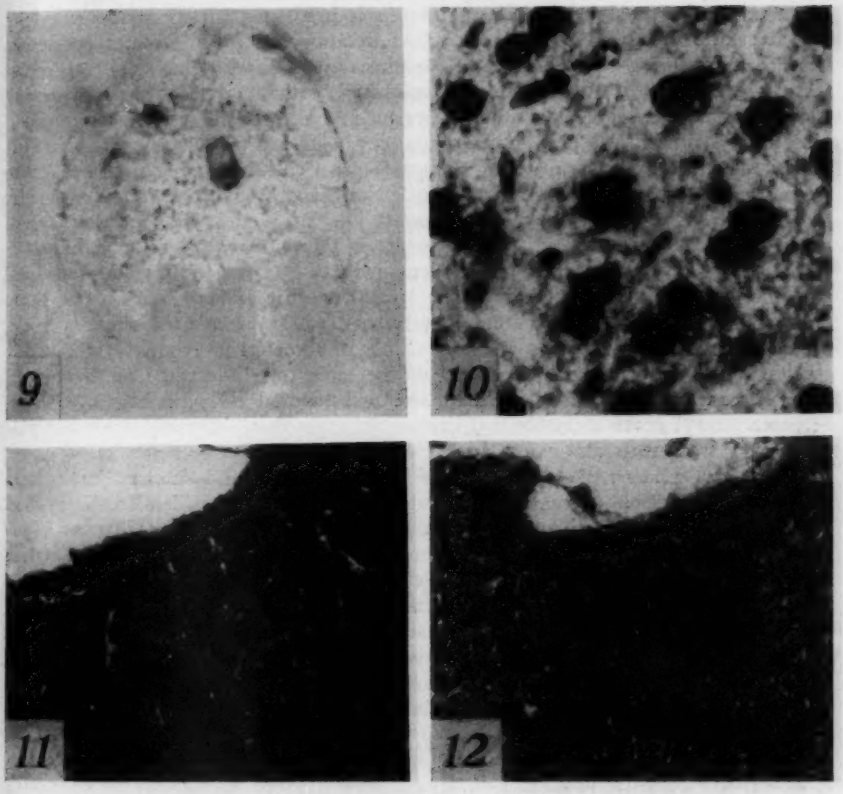

PLATE II. (Photomicrography by Mr. Victor Duran.)

FIGURE 9. Fertilized egg of *Ascaris megalocephala*, Carnoy's, 15 μ section, $\times$ 750; stained with methyl green; Wratten F filter.

FIGURE 10. Mouse liver, as in Figure 2, but first acetylated and then stained with methyl green.

FIGURES 11 and 12. Mouse liver, $\times$ 220; stained with fast green at pH 2; Control (Fig. 11), and after acetylation (Fig. 12).

All these materials stained with methyl green. Fixed smears of isolated cell particulates exhibit staining differences which correspond to those found in histological sections: liver nuclei stain with methyl green and azure, while mitochondria and microsomes stain only with azure but not with methyl green.

If lack or presence of methyl green stainability is not a property of the nucleic acid itself, as these model experiments seem to suggest, a possible clue for the

FIGURES 1 and 2: liver; FIGURES 3 and 4: pancreas; FIGURES 5 and 6: submaxillary gland; FIGURES 7 and 8: motor neurons. Odd numbered figures: stained with azure B; Wratten G filter. Even numbered figures: stained with methyl green; Wratten F filter.

explanation of the observed staining differences in tissues is perhaps provided by the effect of formalin. As mentioned above, fixation in strong formalin (or treatment of Carnoy-fixed material with 50% formalin for three hours) causes loss of methyl green specificity for nuclei. Formaldehyde is known to combine with amino groups of proteins. This reaction could result in making available, for combination with the dye, nucleic acid phosphate groups previously masked by the protein. This hypothesis was tested by subjecting sections to two chemical procedures known to affect amino groups of proteins, the Van Slyke reaction and acetylation by acetic anhydride (see Olcott and Fraenkel-Conrat, 1947). Under the conditions used, these two treatments produce similar results. Their immediate effect consists in a decrease of acidophilia which can be demonstrated by comparing sections stained with fast green at pH 2 before and after treatment (Figs. 11 and 12). The same effect was described by Monné and Slautterback (1951) who used similar procedures. While these reactions result in a loss of some basic groups available for combination with acid dyes, they may at the same time affect the methyl green stainability of nucleic acids; Figure 10 demonstrates that RNA in liver cytoplasm becomes methyl green stainable and that nuclei stain more intensely after acetylation. The treatments also cause the nucleolus in neurons to become methyl green stainable and enhance the stainability of Nissl substance and submaxillary gland cytoplasm and nuclei. Smears of isolated liver mitochondria and microsomes also become methyl green stainable after either treatment. The stainability of ribonucleic acid with azure in any of the mentioned materials is not increased further by these treatments and they do not affect the Feulgen stainability of nuclei.

For unknown reasons the strongly basophilic cytoplasm of the exocrine cells in pancreas does not become methyl green stainable after acetylation or Van Slyke reaction. This probably reflects a fundamental difference in the structure of the nucleoproteins as compared to those of liver cells. Differences in the nucleoproteins of various tissues with respect to their solubility have been described by Koenig and Stahlecker (1952).

Digestion of sections with trypsin in aqueous solution as described by Kaufmann *et al.* (1950) does not produce cytoplasmic methyl green stainability in liver and pancreas. Since the azure stainability was decreased after trypsin digestion, it appears that the enzyme removed the whole nucleoprotein complex in these materials.

The qualitative observations on cytoplasmic staining reported so far indicate that RNA in different tissues may be methyl green stainable or not, and suggest that this is a matter of absence or presence of protein interference. The chemical treatments used bring out further differences in that they may cause an unmasking of stainable groups in some materials but not in others.

2. Nuclear basophilia

As far as nuclear staining is concerned, our own data confirm those of Pollister and Leuchtenberger (1949) relating to the reproducibility of the methyl green procedure. Samples of 20 photometric measurements of the amounts of methyl green bound by diploid nuclei of the mouse pancreas were measured on different slides, prepared and stained at different times. The mean values of methyl green content per nucleus thus obtained were quite similar: 100 ± 4, 104 ± 4, and 111 ± 4 (see Table III).

A comparison of the methyl green and Feulgen stainability of tetraploid and diploid nuclei in the liver and pancreas of the mouse was also made and the data are presented in Table I.

Recent work by Ris and Mirsky (1949), Swift (1950) and Leuchtenberger, Vendrely and Vendrely (1951) has demonstrated that photometric Feulgen dye determinations, if done correctly, provide a reliable measure of the DNA content and degree of ploidy of a nucleus. The present data indicate that this is also true for methyl green staining in these two tissues of the same animal, since very similar Feulgen/methyl green ratios are obtained. The average Feulgen/methyl green ratio of these four types of nuclei is therefore a characteristic constant of 2.56 ± 0.07 for these tissues. Measurements conducted under the same conditions on 25 onion root tip nuclei produced a very different Feulgen/methyl green ratio of 1.45 ± 0.03. This indicates that the dye-binding characteristics of this material are not the same as in the previous case.

TABLE I

Amounts of DNA in arbitrary units of Feulgen dye and methyl green in different nuclei*

	Type of nucleus	Number measured	Average amount Feulgen	Average amount methyl green	Ratio Feulgen/methyl green
Mouse pancreas	2n	25	277± 6	100±4	2.77
	4n	15	533±16	212±8	2.52
Mouse liver	2n	15	275± 6	112±4	2.46
	4n	25	597±11	239±6	2.50
					average 2.56±0.07
Onion root tip		25	—	—	average 1.45±0.03**

* To facilitate comparison, the methyl green value for diploid pancreas nuclei obtained in one experiment is set equal to 100; all other values are proportional to that.

** Calculated on basis of optical densities measured consecutively on the same nuclei stained first with methyl green and then by the Feulgen reaction. (The Feulgen/methyl green ratio of mouse tissue nuclei calculated on basis of average optical densities is 2.47±0.05, very similar to that given above which is calculated from average amounts of dye per nucleus.)

It remains to be determined whether a change in the Feulgen/methyl green ratio of a nucleus can be correlated with any particular process—such as depolymerization of the nucleic acid.

a) Treatment with acid and alkali

Taft (1951) criticized the depolymerization concept on basis of the following experiment: soaking of sections in dilute acid and alkali (which he assumed would cause depolymerization of the DNA) resulted in a decreased Feulgen stainability without affecting methyl green stainability of nuclei. The only conclusion that can be drawn from such an observation is that methyl green, as it was used in this case, did not stain DNA specifically at all. We have repeated this experiment and obtained different results: treatment with dilute HCl (pH 2.1) for 24 hours at room temperature did not affect the Feulgen reaction but increased methyl green stainability noticeably. The latter may be due to extraction of histone by HCl (Kurnick,

1950b). Treatment with dilute NaOH (pH 8.1, 24 hours, room temperature) had a profound effect: nuclei were swollen, vacuolated and in various stages of disintegration. Strands of Feulgen positive and methyl green stainable chromatin were scattered throughout the cytoplasm. Although chromatin had been partially dissolved and still retained methyl green stainability, this observation does not constitute a test of the depolymerization hypothesis, since alkali at low temperature can decrease viscosity without depolymerizing DNA (see discussion by Davidson, 1950).

b) Enzymatic digestion

The action of desoxyribonuclease results in depolymerization and loss of DNA from sections. The effectiveness of enzymatic digestion depends on the type of fixation and also differs in various materials, depending probably greatly on the compactness of the nuclei. Under the conditions which we used, interphase nuclei in many mouse and Ambystoma tissues were Feulgen and methyl green negative after digestion for 40 minutes at room temperature. Some nuclei in sections of Ambystoma spleen, however, retained Feulgen and methyl green stainability after being exposed to enzyme for as long as 90 minutes at room temperature. Used at low temperature and for a shorter time, the enzyme decreased methyl green stainability more rapidly than Feulgen stainability in mouse liver nuclei (Table II). This effect was also noted by Leuchtenberger, Himes and Pollister (1949).

TABLE II

Effect of desoxyribonuclease (0.15 mg./cc. at pH 7 for 30 minutes at 4° C.) on stainability of tetraploid mouse liver nuclei

	Number measured	Average amount Feulgen	Average amount methyl green	Ratio Feulgen/ methyl green
Control	25	597±11	239±6	2.50
After DNA-ase	15	457±15	142±11	3.72
Reduction by enzyme		23%	41%	

c) Effect of hot water and hot acid hydrolysis

Treatment of sections with hot water and Feulgen hydrolysis (1 N HCl at 60° C. for 12 minutes) removes methyl green stainability of DNA. However these treatments also affect the azure stainability of DNA in a similar way; the effect produced is therefore likely to have a more general basis than the peculiar specificity of methyl green. We observed that basophilia of nucleic acid can be partially restored in these cases by subjecting sections to acetylation, the Van Slyke reaction or trypsin digestion. Photometric measurements on several experimental series are presented in Table III.

All determinations were made on diploid pancreas cells in which the treatments used do not produce cytoplasmic staining which would interfere with nuclear measurements. Acetylation or Van Slyke reaction initially increase nuclear stainability approximately 50%. Exposure to hot water produces a small but significant drop of 15% of the Feulgen values while methyl green stainability is

reduced to the level of the surrounding cytoplasm (about 95% transmission of the incident light). Following hot water treatment either of the protein reactions will restore stainability to an extent at least as great or greater than the initial increase it produces in unheated slides; and if the protein reactions precede hot water treatment they protect from loss of stainability to the same extent. At any stage of these treatments nuclear methyl green stainability is removable by desoxyribo-

TABLE III

Effects of various treatments on methyl green and Feulgen stainability of 2n mouse pancreas nuclei; each value is a mean of 20 measurements

	Methyl green			Feulgen
	Exp. No. 1	Exp. No. 2	Exp. No. 3	Exp. No. 1
Control	100±4	104±4	111±4	277±6
After acetylation	156±8	—	161±3	267±6
After Van Slyke	—	142±4	140±4	—
After hot water	0	0	0	234±4
After hot water and acetylation	86±4	—	68±4	—
After hot water and Van Slyke	—	85±4	71±3	—
After acetylation and hot water	—	—	69±3	—

nuclease but resistant to ribonuclease. The azure stainability of nuclei appears to follow the same pattern in the course of these treatments but loss of stainability is never complete. Photometric measurements were not made. Sections of nucleohistone fibers also show similar effects but are more resistant to loss of basophilia than interphase nuclei; they show a less pronounced decrease after hot water treatment and remain methyl green stainable even after Feulgen hydrolysis.

DISCUSSION AND CONCLUSIONS

It is necessary to consider the validity of various model systems and test tube experiments which have been used to investigate the mechanism of methyl green staining. Obviously any model system represents a simplification of the very complex conditions that obtain in tissues and the observations made on such models must be interpreted with great caution. The conditions under which the model system is tested will also influence the results. This is demonstrated by the data reported by Kurnick (1950a); in one experiment the dye-nucleic acid complex was precipitated with alcohol and ribonucleic acid was found to combine with an appreciable amount of methyl green. When lanthanum was used to precipitate the dye-NA complex, only highly polymerized DNA retained methyl green. Since staining of tissues is not ordinarily performed in presence of lanthanum, the significance of this observation with respect to the histological use of the stain cannot be evaluated.

In our own experiments the various model systems were stained in the same way as sections of fixed tissues. Under these conditions the dye was found to be unable to distinguish RNA from DNA. Although this constitutes no definite proof, it suggests that the specificity of methyl green for DNA which undoubtedly exists

in many tissues is not primarily due to an innate inability of the dye to combine with RNA. Staining experiments on tissues furthermore demonstrate that stainable groups in nucleic acids are made available for dye combination by treatments which are known to affect proteins in definite ways. At this point it is not yet possible to suggest a definite mechanism for the protein interference, since removal of amino groups could affect the nucleoprotein in a number of ways. We know from the work of Kaufmann *et al.* (1951), Mirsky and Ris (1951) and Pollister *et al.* (1951) that masking of stainable groups is a general phenomenon which may affect the staining picture obtained with various dyes. Methyl green seems to differ from other basic dyes mainly in being more sensitive to protein interference.

To account for the behavior of methyl green, Kurnick (1950a) assumed the operation of stereochemical factors. On the basis of *in vitro* experiments he claims that all triphenylmethane dyes with two methyl amino groups, such as methyl green, ethyl green and malachite green, exhibit the same type of selectivity. However one sample of Grübler's malachite green which we tested as a tissue stain did not behave like methyl green : it stained liver cytoplasm as well as nuclei and produced a staining picture similar to the nonspecific methyl violet. Pollister and Leuchtenberger (1949), on the other hand, have pointed out that methyl green carries two positive charges and may therefore require phosphate groups at definite spacings for stable combination. Adopting this view for a possible explanation of methyl green selectivity, we may assume that ribonucleoprotein complexes in many tissues are of a type in which the necessary phosphate groups are masked by protein; but in other cases, such as Nissl substance, methyl green is able to combine either because of the different nature of the complex or because of the presence of some relatively "free" RNA.

DNA in nuclei normally combines to a definite extent with methyl green, resulting in a characteristic Feulgen/methyl green ratio. Changes in this ratio can be brought about in a number of ways and impairment of methyl green stainability is not necessarily related to depolymerization. Even the effect of desoxyribonuclease cannot be interpreted in a clear-cut way. The enzyme may simply remove certain exposed groups necessary for methyl green combination at a faster rate than the many more numerous nucleotides which contribute to the Feulgen reaction. Treatment with hot water and Feulgen hydrolysis affect basophilia of DNA in general and the observations suggest that masking of stainable groups is at least partially responsible. A similar conclusion was reached by Herrmann *et al.* (1950) who found that the toluidine blue binding capacity of RNA is impaired when the nucleic acid is heated in the presence of protein. They attribute this effect, which was observed *in vitro,* to the masking of staining sites by basic groups of protein which had become exposed in the course of heating. In our experiments we have never succeeded, however, in restoring maximum stainability in heat-treated sections. This may be due to a low efficiency of the protein reactions under the conditions used; since there is some loss of Feulgen stainability in the course of heating it is also possible that a small loss of the Feulgen reactive material is responsible for a much greater loss of methyl green stainability. The mammalian nuclei which we used have a notoriously low RNA content and staining of nuclear RNA can hardly play an important role in this case. This is borne out by our observations on the effect of digestion by nucleases. However in other types of material staining of nuclear RNA may be an important variable.

For histological purposes methyl green is often used in combination with pyronin, the well known Unna-Pappenheim mixture. This can be used as a satisfactory qualitative method to distinguish RNA and DNA in many tissues. As a precise and quantitative histochemical technique for DNA determinations, methyl green is much less satisfactory than the Feulgen reaction which appears to be less subject to modifying influences. In combination with the Feulgen reaction, methyl green is a useful tool which serves to characterize a particular nucleoprotein complex in terms of a Feulgen/methyl green ratio. Changes of this ratio are of interest but they cannot be interpreted in a precise way.

Summary

1. The capacity of nucleic acids to combine with basic dyes under various conditions was investigated by means of fixed tissues and model systems. Observations and experiments suggest that the specificity for nuclear DNA which methyl green exhibits in many, but not all, tissues is caused by blockage of stainable groups of ribonucleic acid by protein.

2. Nuclear staining with methyl green is also subject to modification by changes in the degree of protein interference, and impairment of stainability cannot be correlated unequivocally with depolymerization of DNA.

3. Since variable degrees of RNA staining, differences in the extent to which DNA staining is affected by protein interference, and possibly changes in the degree of polymerization of the DNA may all influence the staining intensity of nuclei, methyl green staining is not a very suitable method for quantitative determination of DNA in cell nuclei.

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THE NEUROSECRETORY SYSTEM OF BRACHYURAN CRUSTACEA [1, 2]

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Physiological evidence has accumulated which no longer favors the sinus gland as the site of formation of the molt-inhibiting hormone of Crustacea (Bliss, 1951, 1952; Frost, Saloum and Kleinholz, 1951; Havel and Kleinholz, 1951; Passano, 1951a, 1951b, 1952a, 1952b; Travis, 1951a, 1951b; Welsh, 1951). The present studies, designed originally to correlate anatomical relationships of the neurosecretory organs in the brachyuran, *Gecarcinus lateralis* (Fréminville), with the molt and respiratory data recorded on this animal (Bliss, 1951, 1952), include comparative observations on a variety of crabs. It has become clear that assignment of crustacean endocrine functions primarily to the so-called sinus glands has been an over-simplification of a very complex situation.

Materials and Techniques

Specimens of *Gecarcinus lateralis* were shipped periodically to Cambridge from Bermuda. Most other crabs included in this paper were studied in Bimini, B. W. I., while the first author was a Guest Investigator at the Lerner Marine Laboratory of the American Museum of Natural History.

The characteristic bluish-white hue of the living sinus gland was first noted by Brown (1940), his diagrams of several decapod eyestalks depicting not only the sinus gland but its large bluish-white nerve as well. Both sinus gland and nerve are visible when eyestalks of *Gecarcinus* are dissected either in sea water or in a suitable perfusion fluid. It is possible, after removal of the connective tissue sheath surrounding the ganglia, to trace numerous bluish-white nerve tracts that converge and enter the sinus gland. An intense spot of light reveals the deeper of these tracts.

Accentuation of nerve tracts and bluish-white globules, which have proved to be groups of neurosecretory cells, occurs in eyestalks and brain when a preparation is permitted to stand for some time in the beam of a microscope lamp. Increase in salt concentration due to evaporation of perfusion medium causes tracts and globules already visible to become whiter and others hitherto unseen to be apparent. This effect is accomplished more rapidly by use of concentrated sea water or hypertonic perfusion fluid. Addition of a drop or two of glycerol, which clears the tissue, further intensifies nerve tracts and globules. Careful dissection exposes these tracts as groups of brilliant white fibers.

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A modification of the conventional methylene blue technique for nerve differentiation has proved useful. The eyestalk ganglia and brain, stripped of their connective tissue sheath, are placed in the well of a depression slide, containing about 20 drops of perfusion medium and two drops of 0.2% methylene blue. After several minutes the preparation is examined against a black background in the same amount of perfusion medium and two drops of glycerol. Alternate staining and clearing yields a preparation in which nerve cells appear deep blue and nerve tracts white with blue edges. Since it is known that both neurosecretory and other

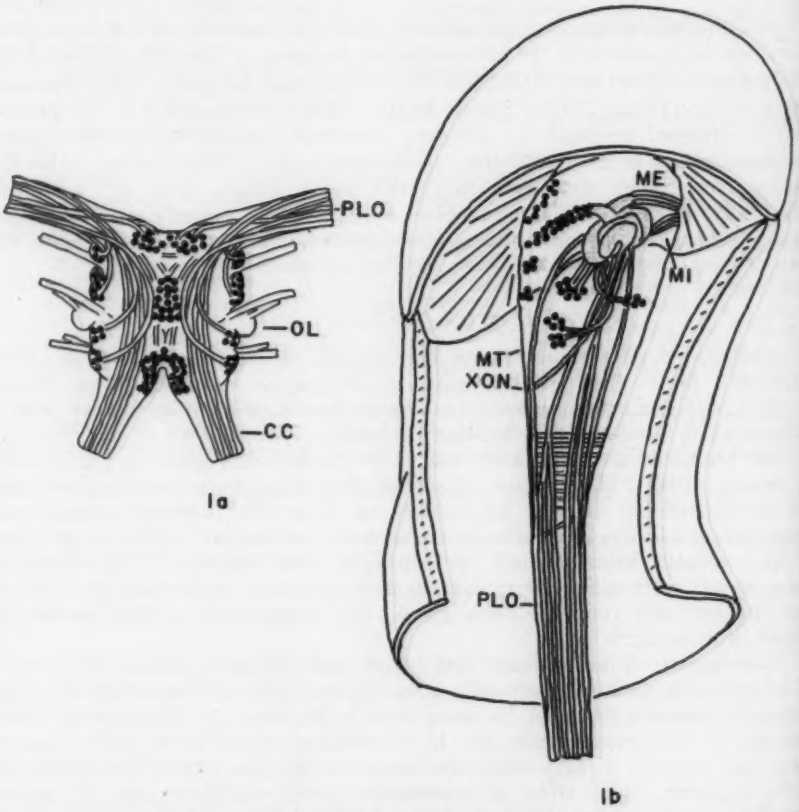

FIGURE 1. Brain (a) and right eyestalk (b) of *Gecarcinus lateralis*, shown in dorsal view when eyestalk is resting in orbit. Circles give location of neurosecretory cell groups. One medial and two lateral groups on the ventral (anterior) face of the brain are not shown. Double lines indicate some of the nerve fiber tracts. Stippled area represents sinus gland. Brain and eyestalk are drawn to the same scale. A considerable portion of the connecting peduncle (PLO) has been omitted. Actual length of adult *Gecarcinus* eyestalk: 0.6–0.7 cm. PLO, peduncle of the optic lobe; OL, olfactory lobe; CC, circumesophageal connective; MT, medulla terminalis; MI, medulla interna; ME, medulla externa; XON, nerve from x-organ.

ganglionic cells accept the stain, these two cell types are then spatially separated by determining in serial sections their relative locations.

Often unstained preparations are best observed in a depression slide against a background of black, white or yellow.

When serial sections are made, tissues are fixed 8 to 12 hours in Helly's, cleared in cedar oil, cut at 6 micra, and stained in chrome-hematoxylin and phloxin, according to Gomori (1941). It has been found that direct immersion of the sections in chrome-hematoxylin without preliminary mordanting in Bouin's results in extraordinarily distinct nerve fibers, whereas after pre-treatment with Bouin's, nerve fibers are relatively indistinct but secretory granules are clearly differentiated.

Observations

Figure 1b shows the right eyestalk of *Gecarcinus lateralis*, as seen in dorsal view with eyestalk in resting position. On casual examination the sinus gland of a freshly-made preparation seems to be innervated by one thick, bluish-white nerve, which is formed at a point proximal to the gland by a branch leading from the ventral portion of the medulla terminalis and one coming from the peduncle of the optic lobe. With application of the several techniques described in the previous section, this nerve is resolved into a number of separate fiber tracts, which intertwine in tortuous fashion as they approach their terminus. Apparent now are many other bluish-white tracts, leading from globules arranged in groups over the surface of the three inner optic ganglia.

The large branch approaching the sinus gland from the proximal ventral portion of the medulla terminalis is the nerve which has been found in crabs by Passano (1951a, 1952a, 1952b) and Bliss (1951, 1952) and in crayfish by Durand (personal communication) to connect the x-organ with the sinus gland. X-organ cells and the nerve which they form are probably identical with the beta cells and the sinus gland nerve of Enami (1951). Bliss and Passano have independently concluded that sinus gland hormone is produced in the x-organ and transported by way of this nerve to the sinus gland. Such a concept is in harmony with that developed for the hypothalamo-hypophyseal system in vertebrates (Scharrer and Scharrer, 1944; Bargmann and Scharrer, 1951; Scharrer, 1952; Palay, 1943, 1945; Smith, 1951), and for the intercerebralis-cardiacum-allatum system in insects (Scharrer and Scharrer, 1944; Scharrer, 1953; Thomsen, 1952).

In Figures 2 and 3 it is shown that, although axons of x-organ cells do form eventually a nerve leading to the sinus gland, the manner in which this is accomplished is quite indirect. The x-organ is composed of two groups of cells, axons of one group forming a tract which descends at once towards the optic lobe peduncle, those of the other group partially circling the medulla terminalis before they head toward the peduncle. Fibers from both tracts now spiral within the peduncle, re-gather in two groups, ascend again to the peduncle, and proceed towards the sinus gland as one large nerve. Evidence for this indirect route has been obtained from glycerine-cleared preparations, both unstained and stained with methylene blue, and from serial sections.

This devious pathway may cause wonder unless it is remembered that the third and fourth optic ganglia are parts of the brain which have, in the course of evolution, been drawn out into the eyestalk. Still pointing towards the center of the

brain, as do axons of many neurosecretory cells within the brain, developing x-organ fibers must turn sharply to reach the sinus gland. Re-orientation may be facilitated by a circling of the optic lobe peduncle. Incidentally there results an increase in the space available for storage of secretory material. Where nerve fibers circle and

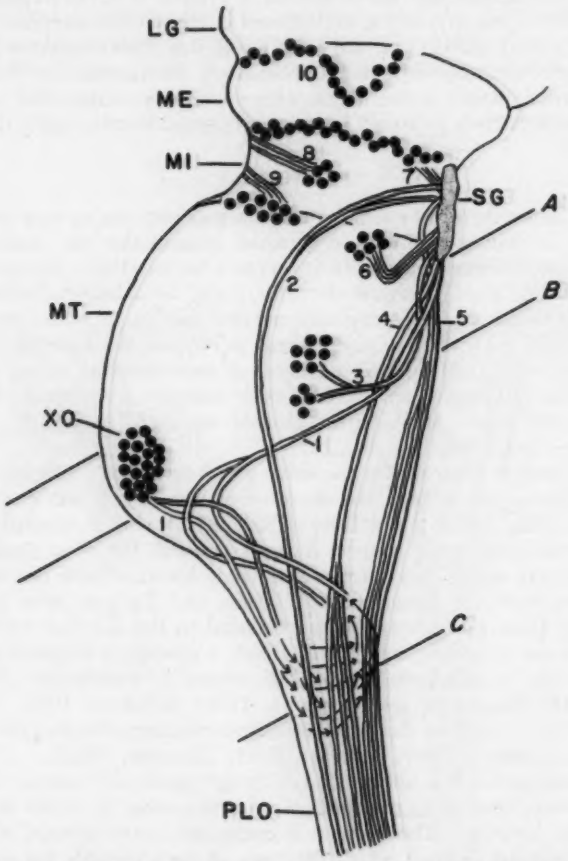

FIGURE 2. The excised right eyestalk ganglia of *Gecarcinus lateralis*, anterior aspect. Circles give location of neurosecretory cell groups. Double lines indicate nerve fiber tracts, which are numbered as in Figure 3. A, B, C designate the three planes of section shown in Figure 3. XO, x-organ; SG, sinus gland; LG, lamina ganglionaris; other abbreviations as in Figure 1.

turn distally towards the sinus gland, secretory material accumulates in such amounts that the axons are almost filled with basophilic granules. This may result from reduced rate of movement in these regions. According to E. Scharrer (personal communication), in vertebrates, likewise, secretory material accumulates at axonal bends and turns.

The original observations that a nerve connects x-organ and sinus gland remain uncontested. The riddle of where the sinus gland nerve runs when, as has been reported often in the literature, it is lost in the medulla terminalis, is solved. This

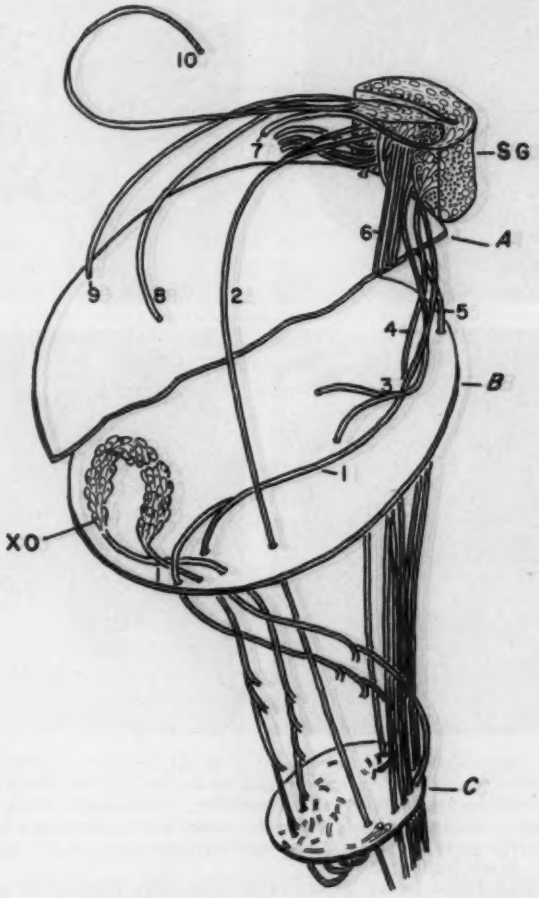

FIGURE 3. Schematic diagram of the x-organ, sinus gland, and fiber tracts of the right eyestalk of *Gecarcinus lateralis*. Fiber tracts and planes of section are designated as in Figure 2. XO, x-organ; SG, sinus gland; 1 (= XON in Figure 1), nerve from x-organ.

nerve has no identity as such before the point where the peduncle-circling fibers converge.

Several groups of bluish-white globules observed proximal to the sinus gland in the living preparation and other groups lying distally (Figs. 1b and 2) are recognizable in chrome-hematoxylin-phloxin stained serial sections as masses of neuro-

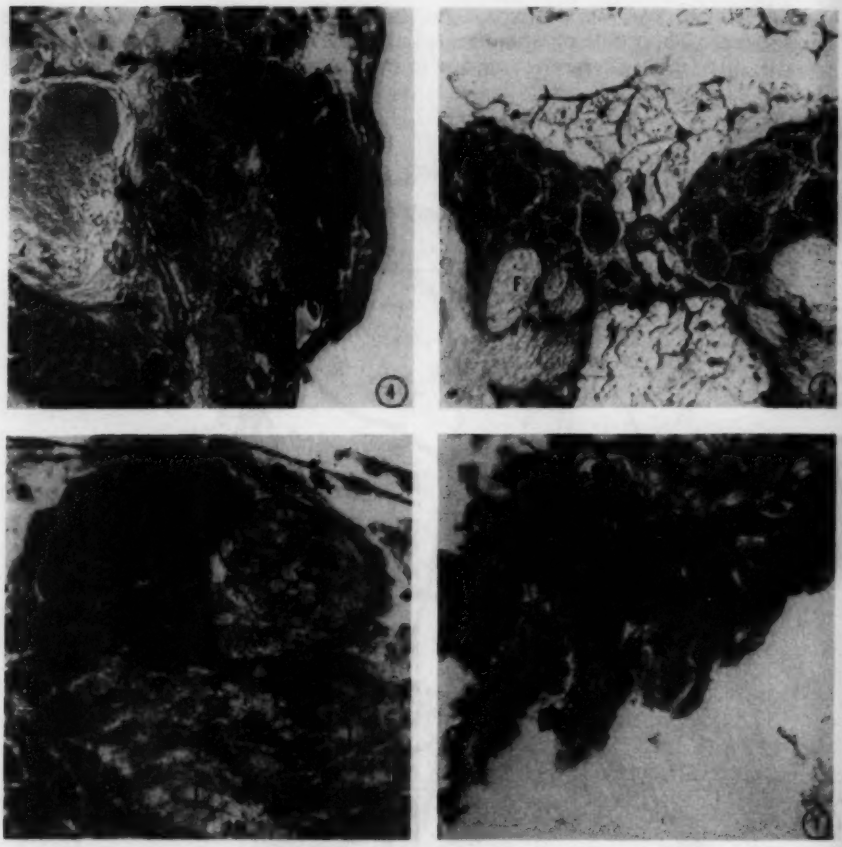

FIGURE 4. Longitudinal section through the sinus gland (right eyestalk of *Gecarcinus lateralis*) in the region where fibers of the x-organ nerve (A) fan out distally to form a sinus gland lobe which has the appearance of an inflorescence. The swollen endings (B) of x-organ axons contain darkly staining secretory material. Distal to the sinus gland are neurosecretory cells (C) and coarse nerve fibers (D) from neurosecretory cells of the medulla interna and medulla externa. Magnification: 200 ×.

FIGURE 5. Two medial frontal groups of neurosecretory cells on the dorsal (posterior) face of the brain of *Gecarcinus lateralis*. In one cell (E) secretory granules fill the axon as well as the cell body. Next to this cell is a large coarse-fibered nerve tract (F) which can be traced in subsequent sections into the optic lobe peduncle. Magnification: 200 ×.

FIGURE 6. Cross section, left eyestalk of *Gecarcinus lateralis*. Included are a small portion of the sinus gland (G), the x-organ nerve fiber tract (H), and fiber tracts (I, J) formed from axons of neurosecretory cells of the brain and medulla terminalis. In x-organ fibers in particular, secretory granules can be seen just inside the fiber membrane. Many granules are visible within the sinus gland. Magnification: 400 ×.

FIGURE 7. A regenerated sinus gland from the right eyestalk of a specimen of *Gecarcinus lateralis* seven and one-half months after both sinus glands had been removed. The regenerated

secretory cells. Coarse lilac-colored axons link cell bodies with the sinus gland. Figure 4 is a longitudinal section showing these coarse axons, the sinus gland, and distal to it large neurosecretory cells. These cells contain concentrically arranged fine granules and irregular inclusions, similar to those described by Hanström (1947) for the cells of the x-organ in Natantia.

Although granules can be seen along an axon for some distance from the secretory cell body, they soon become less numerous or disappear entirely, to appear again in great numbers just before the fiber reaches the sinus gland. The intermediate parts of the fiber contain homogeneous material which stains lilac with chrome-hematoxylin-phloxin. A somewhat analogous situation has been observed in vertebrate hypothalamic-posterior lobe preparations (E. Scharrer, personal communication).

Lying on the surface of the brain (Fig. 1a) are many more neurosecretory cell groups, appearing as collections of bluish globules in the living specimen and as grape-like clusters in serial sections. Figure 5 is a photomicrograph of two medial frontal groups lying on the posterior face of the brain. These neurosecretory cells have the same general characteristics as cells of the insect pars intercerebralis (Scharrer and Scharrer, 1944) and of the vertebrate hypothalamus (Bargmann and Scharrer, 1951; Smith, 1951). They are indistinguishable from neurosecretory cells of the eyestalk of *Gecarcinus* and, like the latter, contain concentrically arranged granular inclusions which stain blue-black with chrome-hematoxylin.

Bluish-white tracts, which appear in serial sections as groups of coarse lilac nerve fibers, run in the living preparation from the globular clusters of the brain into the optic lobe peduncle. Other whitish tracts and, in serial sections, other coarse lilac fibers enter the brain from the circumesophageal connectives and continue out the optic lobe peduncle. Since the thoracic ganglionic mass of *Gecarcinus*, like that of *Sesarma* (Enami, 1951), contains many giant neurosecretory cells, the sinus gland in the eyestalk of a crab appears to be the terminus for axons originating as far away as the thorax. Clearly necessary is a reconsideration of the structure and function of the sinus gland.

Figure 6 shows in cross section the sinus gland and the nerve from the x-organ. Lying just within the membrane of each nerve fiber are granules and granular aggregates, strikingly similar to those found within the cell bodies of these axons and within the sinus gland. In Figure 4 this nerve is seen to be not merely entering but actually forming a portion of the sinus gland. Its fibers, gradually expanding as they terminate to produce the effect of an inflorescence, contain within their bulbous endings acidophilic secretory material and basophilic granules. To the upper left of the sinus gland, axons from neurosecretory cells of the medulla externa approach laterally, to form another section of the sinus gland. Numerous microscopic sections indicate that the sinus gland of *Gecarcinus* is a mass of swollen nerve fiber endings, grouped into lobes according to the fiber tracts from which they arise. This has been represented schematically in Figure 3.

Support for this concept of an organ which previously had been considered glandular in its own right came from regenerated sinus glands. Five weeks after

structure is characterized by small size and abnormal position but has essentially normal staining properties. Magnification: 400×. All photomicrographs made from tissues fixed in Helly's, cut at 6 micra, and stained with chrome-hematoxylin-phloxin.

bilateral sinus gland removal the eyestalks of three crabs contained no sign of sinus glands in their normal position. Near the x-organ, just under the connective tissue sheath covering the medulla terminalis of each eyestalk, considerable bluish-white secretory material had accumulated. Fixed and stained with chrome-hematoxylin-phloxin, similar secretory material from a crab, seven and one-half months after bilateral sinus gland removal, resembled histologically (Fig. 7) that found in a normal sinus gland. Fibers from neurosecretory cells of the eyestalk and brain could be traced into this regenerated sinus gland.

Twenty-two days after the x-organ nerve and part of the x-organ itself had been removed from each eyestalk of a crab, one eyestalk retained no sign and the other eyestalk only faint traces of a normal sinus gland. Fibers of remaining x-organ cells and axons from the optic lobe peduncle together had produced on

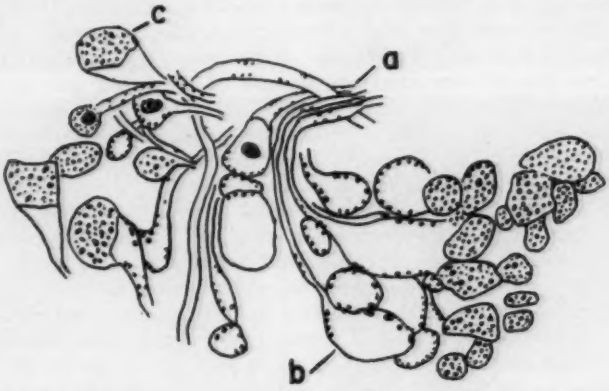

FIGURE 8. A portion of a regenerating sinus gland (right eyestalk of *Gecarcinus lateralis*) which appeared as illustrated after partial removal of both x-organs and x-organ nerves three weeks before had caused degeneration of the normal sinus glands. Secretory granules line the nerve fibers (a) and their swollen endings (b). Many granules have accumulated within masses of acidophilic secretory material (c) which lie at the tips of the bulbous nerve endings.

the medulla terminalis next to the x-organ a regenerating sinus gland, part of which is sketched in Figure 8. Bulbous nerve endings and their acidophilic contents, in which were included basophilic granules, appeared in the same sort of inflorescence as is typical of the normal sinus gland (Fig. 4). Granules lined the nerve fibers and their swollen endings.

Removal of a normal sinus gland seems to cause partial degeneration of neurosecretory cell axons. Upon subsequent regeneration they reach the surface of the ganglion at a point close to the x-organ, where they form a structure in many ways resembling the original sinus gland. Certain respiratory data (Bliss, 1952) suggest that although regenerated sinus glands of *Gecarcinus* continue to release secretory material, the mechanism of release is not normal.

Of considerable interest are the recent reports that regenerated sinus glands are formed in several species of crabs not only after sinus gland removal but also after

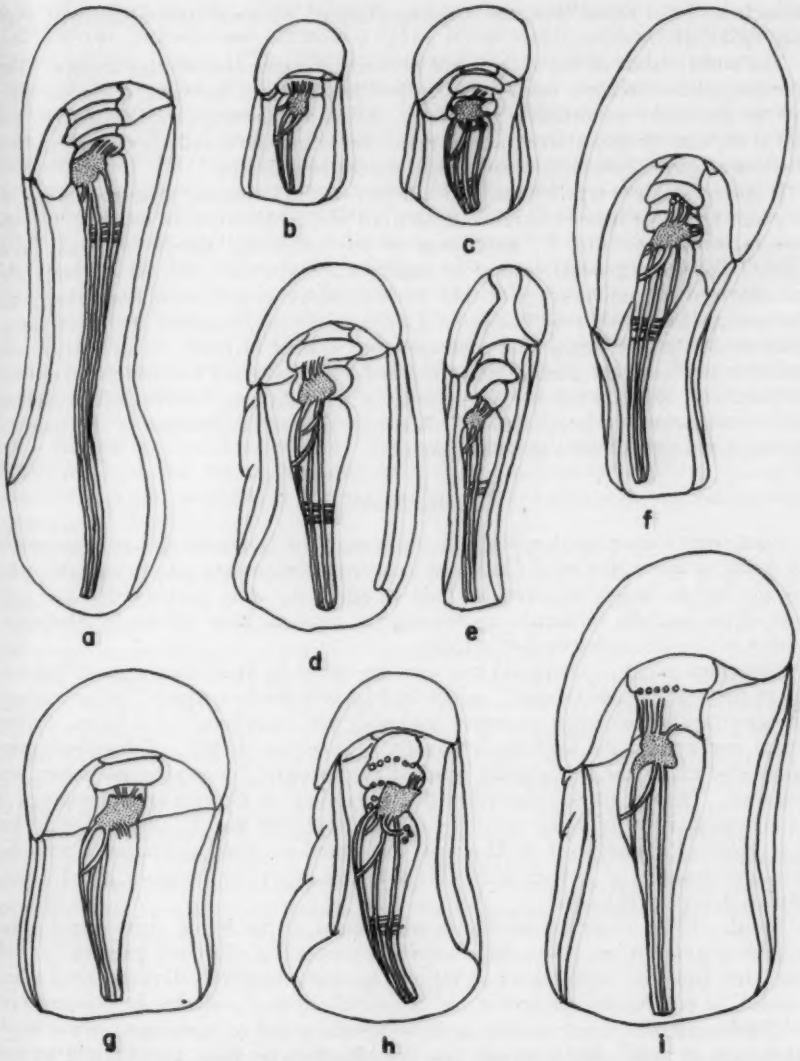

FIGURE 9. Right eyestalks of brachyurans. (a) *Cardiosoma guanhumi;* (b) *Pachygrapsus transversus;* (c) *Sesarma (Holometopus) ricordi;* (d) *Callinectes ornatus;* (e) *Uca mordax;* (f) *Ocypode albicans;* (g) *Portunus (Achelous) spinimanus;* (h) *Carcinides maenas;* (i) *Grapsus grapsus.* The eyestalks of *Callinectes exasperatus* are similar to those of *C. ornatus.* Stippled areas represent sinus glands. With one exception (*Uca mordax,* 3½ ×), all drawings are magnified approximately 2½ times as much as is the *Cardiosoma* drawing. Actual length of adult *Cardiosoma* eyestalk: 2–2½ centimeters.

transection of the nerve from the x-organ (Enami, personal communication; Passano, 1951a, 1952b).

The sinus glands of *Gecarcinus* are receiving centers and storage depots. For some unexplained reason, conceivably related to control of hormone release or perhaps to activation of inactive precursors, secretory products synthesized in the central nervous system are carried by nerve fibers to their swollen endings, the so-called sinus gland, before being released into the hemolymph.

Is this complex synthesizing-transporting-storing-releasing system unique to *Gecarcinus* or does it exist in other Brachyura and in Macrura as well? Observations on eyestalks (Fig. 9) and brain of ten additional species of crabs have revealed the same general pattern of bluish-white globules and nerve tracts and have affirmed the universality of this neurosecretory system in tail-less decapods. The eyestalk and brain morphology of *Cambarus*, to be presented in a later paper, demonstrates the existence of a corresponding system in tailed forms. It is now justifiable to extend the concept first proposed for vertebrates and insects (Scharrer and Scharrer, 1944) to another large group of invertebrates, the decapod Crustacea, and to underscore the possibility that basically similar neurosecretory mechanisms may exist throughout the animal kingdom.

Discussion

Ever since Hanström described the x-organ of the Decapoda, it has been sought and found in many species of Crustacea. Its secretory nature was recognized cytologically but no endocrine function could be attributed to it, particularly since portions of the medulla terminalis containing the x-organ were shown by Hanström to have no chromatophorotropic activity.

The sinus gland of Decapoda has been described by Hanström as a differentiation of the neurilemma (sheath), which had become thick, syncytial, full of radially arranged canals containing secretory material, and innervated by a large, coarse-fibered nerve from the medulla terminalis (Hanström, 1939). Subsequent cytological studies on the sinus gland resulted in confusion concerning its nature and structure. Dethier (1942) described the sinus gland of *Cambarus* as composed of anastomosing rows of cells, yet Pyle (1943) observed no cell boundaries in the sinus gland of *Pinnotheres* or *Homarus* and noted its distal migration during the larval development of *Pinnotheres*. He asked in effect: is the sinus gland a non-cellular storage syncytium?

Smith (1948) observed the bluish-white color of the living sinus gland nerve and the presence of an eosinophilic secretion product in the fixed preparation. A year later, from his experiments on retinal pigment migration following sinus gland removal, he concluded that production of retinal pigment activator in *Hemigrapsus* and *Pachygrapsus* occurs not only in the sinus gland but in many parts of the nervous system as well. For a second time the idea that the sinus gland might be specialized for storage was proposed, Smith suggesting in addition the possibility that the organ might facilitate periodic hormone release. Bowman (1949) found the results of chromatophore experiments in *Hemigrapsus* understandable when the highly active sinus gland was considered a storage-release organ for material produced by chromatophorotropically-active regions of the central nervous system.

The recent observations that eyestalk and x-organ removals, but not sinus gland

removal, induce molting or physiological changes associated with molt (Bliss, 1951, 1952; Frost, Saloum and Kleinholz, 1951; Havel and Kleinholz, 1951; Passano, 1951b, 1952b; Travis, 1951a, 1951b; Welsh, 1951), coupled with microscopic evidence of the presence of secretory material along the nerve connecting x-organ (or beta cells) with the sinus gland (Bliss, 1951, 1952; Durand, personal communication; Enami, 1951; Passano, 1951a, 1952a, 1952b) and along nerves from other neurosecretory cell groups in the eyestalk and brain, have led to the hypothesis presented in this paper, namely, that a large neurosecretory system, involving many parts of the central nervous system, exists in decapod crustaceans, and that the sinus gland is primarily, and perhaps exclusively, a storage and release site for material produced within this neurosecretory system. The initial suggestions made by Pyle, Smith and Bowman have been shown to be justified.

Before concluding, the authors wish to make brief comment on a paper by Enami (1951). After a series of careful histological studies on the eyestalks, brain, and thoracic ganglia, in which the neurosecretory cells of three species of *Sesarma* were mapped, Enami has concluded that the sinus gland and the neurilemma of this organ carry on nuclear secretion. Nothing the present writers have seen has indicated such a process. They suggest that the bulbous nerve endings which make up the sinus gland may have been interpreted by Enami as nuclear capsules and the acidophilic material within those endings as nuclear secretion products.

If the organ which has been named the sinus gland releases but does not synthesize products of secretion, should it still be known by that name? It is an integral part of a diffuse neurosecretory complex, composed of cell bodies, axons, and their swollen endings. It is the portion of this complex which accomplishes the essential processes of storage, possibly of transformation, and of release.

What purposes are served by a diffuse neurosecretory system in contrast to a discrete compact gland? This question can be answered only as physiological data accumulate, but certain possibilities can be suggested. With a large part of the central nervous system occupied by secretory cells and their fiber tracts, it is likely that their specialization for neurosecretion has not eliminated their capacity to act as conductors of nerve impulses. A neurosecretory cell group could then trigger the release of its own secretory material, by conducting impulses to its endings in the storage site. If formed from completely independent groups of neurosecretory cells, the sinus gland might serve as the storage-release center for more than one hormone (see Brown, 1944, 1951; Brown, Sandeen and Webb, 1951; Brown and Hines, 1952; Scharrer, 1953).

Summary

1. The land crab, *Gecarcinus lateralis* (Fréminville), was selected as the principal subject for this study of anatomical relationships existing between neurosecretory centers in the eyestalks and brain of brachyuran Crustacea. Observations were also made on ten other species of crabs.

2. Techniques useful in clarifying crustacean endocrine structures have been described.

3. It has been found that the sinus gland is actually a mass of swollen nerve fiber endings, arranged in the form of an inflorescence and bearing secretory

material. The histological structure of regenerated sinus glands, which appear after sinus gland removal, resembles that of normal sinus glands.

4. Nerve fibers, the endings of which compose the sinus gland, originate in neurosecretory cells of the brain, the eyestalk ganglia, and possibly the thoracic ganglionic mass. X-organ fibers contribute their endings to the sinus gland.

5. Neurosecretory products of the living preparation appear as bluish-white material in the cell bodies, nerve tracts, and sinus gland. When stained with Gomori's chrome-hematoxylin-phloxin, neurosecretory material assumes a homogeneous acidophilic or granular basophilic form.

6. There is developed the concept of a large neurosecretory system involving the brain, cells of the eyestalk ganglia, and perhaps those of the thoracic ganglion, all of which transmit their secretory products to storage-release organs, the so-called sinus glands.

7. The similarities between this system in decapod crustaceans, the hypothalamo-hypophyseal system in vertebrates, and the intercerebralis-cardiacum-allatum system in insects are emphasized. It seems justifiable to extend to the decapod crustaceans the concept first proposed for vertebrates and insects by Scharrer and Scharrer (1944).

8. Recent literature on the histology and physiology of the sinus gland and x-organ is reviewed and interpreted in terms of the hypothesis, proposed in this paper, of a crustacean neurosecretory system.

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EFFECTS OF X-RAYS UPON HAPLOID AND DIPLOID EMBRYOS OF HABROBRACON [1]

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Correlations between radiosensitivity and ploidy, the number of chromosome sets, have been made by Stadler (1929), Müntzing (1941), Fröier, Gelin and Gustafsson (1941) and Smith (1943, 1946) for cereals; by Latarjet and Ephrussi (1949) for the yeast, Saccaromyces; by Whiting and Bostian (1931), Clark and Kelly (1950), and Clark and Mitchell (1951) for Habrobracon; and by Lamy and Muller (1939) for Drosophila. These studies, with the exception of the last, have been consistent in demonstrating, for the material tested, that there is a greater resistance with higher ploidy and the results have been taken as evidence that x-radiation damage is primarily chromosomal. That there is less chance for homozygous deficiencies to occur in polyploids than in diploids and that all deficiencies in haploids would be lethal, have been given as explanations. Lamy and Muller found that diploid and triploid Drosophila x-rayed as embryos are equally radiosensitive and they assume that the deleterious effects in this case are largely non-chromosomal ("physiological").

The senior author and associates have been studying the effects of x-rays on haploids and diploids of the parasitic wasp, Habrobracon, during different stages of its life cycle in order to determine to what extent genome number can be correlated with radiosensitivity. Comparison of radiosensitivity during the pupal, prepupal and larval stages has shown that diploids are more resistant than haploids. The present paper reports on radiosensitivity during the early embryonic stages.

Material and Methods

In Habrobracon, haploid males arise from unfertilized eggs and diploids (male and female) from fertilized eggs. Whiting (1943) has established the sex-determining mechanism and has shown that cultures can be obtained that contain (1) only haploid males, (2) haploid males and diploid females, (3) haploid males, diploid females and diploid males. In the present study comparison is made between (1) and (2). Stocks No. 33 and No. 17-o[1] (ivory) were used. Cultures of haploid embryos were obtained from No. 33 unmated mothers while cultures of haploid and diploid embryos ("mixed" cultures) were obtained from No. 33 females mated to No. 17-o[1] males. Progeny of mated females normally consist of about 60 per cent diploids and 40 per cent haploids.

Embryos of known ages were x-rayed. They were then (a) placed in syracuse dishes containing a mineral oil ("Nujol") and observed for hatchability or (b)

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placed upon food (paralyzed Ephestia larvae) in order that survivors could develop into adults. All cultures were kept at 30° C. At this temperature eggs hatch about 30 hours after being laid. The larvae in mineral oil were counted 40 hours after oviposition and the incidence of hatchability noted. Eggs placed upon Ephestia larvae were observed at various stages of development and comparison of sex ratios was made between control and treated cultures from mated females.

All microscopical studies were made upon whole mounts of embryos fixed in Kahle's fluid and stained with the Rafalko modification of the Feulgen technique.

For x-ray experiments a dual-tube self-rectifying outfit with a simultaneous cross firing technique was used. The secondary voltage was 182 kv.; the tube was 25 ma.; the output intensity was 110 r per minute. There was an inherent filter equivalent to 0.2 mm. copper. X-radiation was obtained at the Marine Biological Laboratory, Woods Hole, Massachusetts.

Results

Embryos treated between one and three hours of age

As determined by microscopical study of whole mounts, untreated embryos between one and three hours of age were found not to have progressed beyond cleavage. There is a syncytium at this stage with the nuclei rapidly undergoing synchronous mitosis. For the majority of the eggs the number of nuclei found was over fifty. A range from the pronuclear stage to embryonic stages having over

Table I

Hatchability ratios for eggs from mated and unmated females
(Age of embryos when x-rayed, 1-3 hours)

Dose (r)	Haploids (from unmated ♀ ♀)		Haploids	Diploids*	Diploids (estimated) hatchability
			(from mated ♀ ♀)		
	No. eggs	Hatchability	No. eggs	Hatchability	
0	573	.93±.01	1071	.92±.01	.91±.01
27.5	91	.97±.02	131	.93±.02	.90±.04
55	148	.91±.02	263	.87±.02	.75±.03
110	263	.85±.02	433	.47±.02	.22±.03
165	220	.69±.03	168	.31±.03	.07±.02
220	331	.51±.03	319	.18±.02	.00
275	137	.27±.04	146	.05±.02	
330	195	.10±.02	225	.04±.02	
440	142	.03±.01	161	.02±.01	

* Adult female ratio in controls .60 (729/1226 adults).

250 nuclei was observed but these extremes were exceptional. Eggs from mated and unmated females were in the same stages of development.

Hatchability ratios for eggs of this age at time of treatment demonstrate that those from mated females are more sensitive than those from unmated (Table I). This difference is clearly shown after doses from 110 r to 275 r. For example,

after treatment with 110 r, the hatchability for eggs from mated females is 0.47 ± .02 as compared with 0.85 ± .02 for eggs from unmated mothers. Untreated eggs from mated and unmated females do not differ in hatchability (Table I). Since the incidence of female offspring from mated control mothers was found to be 0.60 on the basis of 1226 adults counted, these data can be used for deriving the hatchability of diploid eggs (Table I).

Since the hatchability data (Table I) indicate that haploids are more radioresistant, the ratio of adult females $\left(\frac{♀♀}{\text{Total}}\right)$ from x-rayed eggs of mated mothers should be lower than from control eggs. This is substantiated when comparison is made of the ratios of haploid males and diploid females from x-rayed and control cultures (Table II). At all doses there is a marked decrease in the ratio of females. For example, after treatment with 220 r, 33 males and 6 females were obtained (0.15 ± .06) as compared with 46 males and 60 females in the untreated group (0.57 ± .05).

Reference to Table II will show that there is a decrease in hatchability with increase in dose and that this occurs for eggs from both mated and unmated females.

TABLE II

Hatchability and eclosion ratios for offspring from mated females (Age of embryos when x-rayed, 1–3 hours)

Dose (r)	No. Eggs	No. adults		Larvae / Eggs	Adults / Larvae	♀♀ / Total
		♂♂	♀♀			
0	146	46	60	.84±.03	.86±.03	.57±.05
55	116	43	43	.81±.04	.91±.03	.50±.05
110	101	26	7	.55±.05	.59±.07	.21±.07
165	284	42	8	.18±.02	.98±.02	.16±.05
220	404	33	6	.11±.02	.86±.05	.15±.06

Once the eggs have hatched, however, there appear to be no further deleterious effects as shown by comparison of ratios of adults/larvae at different doses. There is no developmental lag in survivors and the adults show no structural abnormalities. Death due to irradiation occurs before hatching.

Embryos that were x-rayed (220 r) during cleavage (one–three hours of age) were fixed at an age when somites were normally present (12–15 hours of age) in the control embryos. Examination of whole mounts showed that some of these treated embryos had formed somites and appeared normal. The majority, however, were either in cleavage or early blastema. Nuclei in the cleavage stages were in interphase and were very much enlarged, being up to four times the diameter of untreated nuclei. Many were clumped together forming dark patches within the egg. The large number of nuclei in treated embryos which had died may suggest that cleavage continued after x-radiation. In some eggs, nuclei could not be found, due, perhaps, to disintegration of chromatin material. A sufficient number of eggs from haploid and mixed cultures was not prepared to make a quantitative microscopical comparison between these groups.

TABLE III

Hatchability and eclosion ratios for offspring from mated females (Age of embryos when x-rayed, 3–4 hours)

Dose (r)	No. eggs	No. adults		Larvae / Eggs	Adults / Larvae	♀♀ / Total
		♂♂	♀♀			
0	197	53	102	.89±.02	.89±.02	.66±.04
220	442	48	81	.34±.02	.86±.03	.63±.04

Embryos treated between three and four hours of age

Embryos of three–four hours of age from mated mothers were treated with 220 r and were allowed to develop into adults. The sex ratios of adults emerging were compared with those from control eggs (Table III). The incidence of females from control and treated eggs is not significantly different. This indicates that haploids and diploids are equally radiosensitive when treated at this stage of development. There are no developmental effects of an injurious nature after hatching as shown by the ratios of adults/larvae. Most of the three-hour old control embryos have completed cleavage and have hundreds of nuclei within the egg. These nuclei are migrating or have migrated to the periphery of the egg (blastema stage).

Embryos treated between 13 and 15 hours of age

Older embryos in the stage when somites are present that are treated with x-rays may not show injury during the egg stages. However, deleterious effects may be seen during later stages of development. Embryos, 13–15 hours of age, were x-rayed with either 722 r or 1444 r. Comparison of the number of larvae obtained showed that there is no decrease in hatchability over the controls (Table IV). The number of adults, however, is markedly decreased for those treated with 1444 r. These groups tended to show a lag in development as larvae and to remain as larvae after the controls had become adults. Some of the larvae did not grow to full size, but continued development to the pupal stage without spinning a cocoon.

TABLE IV

Hatchability and eclosion ratios for offspring from mated and unmated females (Age of embryos when x-rayed, 13–15 hours)

Dose (r)	Mothers	No. eggs	No. larvae	No. adults		No. dying as larvae	Larvae / Eggs	Adults / Larvae
				♂♂	♀♀			
0	mated	42	30	11	12	2	.71	.77
722	mated	80	64	27	20	8	.80	.73
1444	mated	53	40	4	3	25	.75	.17
0	unmated	42	39	35		4	.93	.90
722	unmated	47	43	36		7	.92	.84
1444	unmated	50	44	12		24	.88	.27

There are insufficient data to state whether haploids and diploids are affected differentially when irradiated at 13–15 hours of age.

Discussion

Comparisons of radiosensitivity among individuals differing in ploidy have appeared in the literature in an attempt to evaluate the extent of chromosomal ("genetic") and cytoplasmic ("physiological") injury. Since differences in the number of chromosome sets are the most obvious distinguishing characteristics, the occurrence of a differential lethal effect has been taken as a criterion that nuclear injury has taken place. It is less obvious, but pertinent, that different metabolic conditions may exist in diploids and polyploids. Investigations herein reported of x-ray effects for haploids and diploids of Habrobracon suggest that radiosensitivity cannot be correlated with genome number at all stages in the life cycle. The differential radiosensitivity between haploids and diploids depends upon the stage of development at which they are irradiated. Haploids are more resistant than diploids during cleavage, equally resistant immediately following cleavage, and less resistant than diploids during the larval, prepupal, and pupal stages. One might inquire, therefore, if a differential effect between diploids and polyploids is an adequate criterion for distinguishing between chromosomal and cytoplasmic injury.

Most of the investigators who have reported a differential effect upon survival have found that the individuals with the greater number of chromosome sets are more resistant. Some of them (Müntzing, 1941; Latarjet and Ephrussi, 1949; Clark and Kelly, 1950; Clark and Mitchell, 1951) have suggested that (1) chromosome breakage occurs with the resultant loss of acentric fragments, and (2) there is a greater chance for individuals with higher ploidy to retain unbroken chromosomes that could compensate for the homologous fragments that are lost.

There are some observations that are difficult to reconcile with interpretations of radiation injury purely in terms of a chromosome breakage hypothesis. Studies on diploid and polyploid cereals have correlated gross observations on survival with the number of chromosome breaks (Fröier, Gelin and Gustafsson, 1941; Smith, 1943, 1946). These investigators have shown that polyploids, even though more resistant than diploids, have more anaphase bridges and chromosome fragments than comparable diploids. Fröier, Gelin and Gustafsson (1941) have shown that the germination and sprouting ability of polyploids were unimpaired even when 50 per cent of the mitoses had bridges and fragments, but the growth of the diploids was impaired with much less nuclear disturbance. Further, they showed that at high doses (50,000–60,000 r) nuclei of *Avena sativa*, a hexaploid, are still able to divide even if the chromosomes are fragmented to the extent of discontinuity. Marshak and Bradley (1944) have shown that mitotic inhibition is inversely proportional to the number of chromosomes, but independent of chromosome length. They suggest that this indicates an effect upon the centromeres.

If a chromosomal basis for injury is to be postulated, then a different mechanism must be used to explain the fact that haploid embryos of Habrobracon are more resistant than diploids when treated during cleavage. The observations by Fröier, Gelin and Gustafsson (1941) and Smith (1943, 1946) on cereals and by Bishop (1950) on Tradescantia, that the number of chromosome breaks following x-radiation is proportional to the number of chromosomes, would suggest that the

diploid nuclei of Habrobracon incur twice as many chromosome breaks as comparable haploid nuclei. One might reason that the diploids would be more sensitive than the haploids because they have received twice as much injury per nucleus. It has already been noted, however, that polyploids with more chromosome breaks per nucleus are more resistant than diploids with fewer chromosome breaks per nucleus. Differences between the stages of development that allow haploids to be more resistant at one stage and more sensitive at another should perhaps be considered. A number of obvious differences exist between the cleavage stage and the later post-embryonic stages of Habrobracon. During cleavage there is a syncytium with the nuclei dividing rapidly and synchronously throughout the embryo while in later embryonic and the post-embryonic stages cells are present with active proliferation being restricted to localized regions. There is little differentiation in the embryos during cleavage. Whether or not these differences have anything to do with the reversal in radiosensitivity is not known.

If differential radiosensitivity is to be taken as a criterion of chromosomal injury, then the equivalent radiosensitivity of haploid and diploid embryos when x-rayed immediately after cleavage must be taken to mean that extra-chromosomal factors are involved. The radioresistance is increasing rapidly at this time and, therefore, a slight lag in development between haploids and diploids could result in a large difference in radiosensitivity. Kelly (unpublished), using 50 per cent hatchability as the criterion for comparative lethality, has shown that haploid embryos increase in resistance from a dose of about 200 r during cleavage to 7000 r in about four hours. Lamy and Muller (1939) found that diploid and triploid Drosophila when treated as embryos were equally radiosensitive. They explained this by assuming that death was due to a "non-genetic" ("physiological") type of injury. The data reported in the present paper suggest that the Drosophila embryos were x-rayed after cleavage.

Embryos x-rayed during cleavage or in early blastema are arrested in development during the egg state or not at all. Post-embryonic development is normal. Embryos x-rayed at a later stage, however, may show a post-embryonic lag in development and become arrested at the larval stage. Dent and Amy (1950) treated embryos and larvae of Habrobracon with P^{32}. They found a lag and an arresting of development at the larval stage. Henshaw and Henshaw (1933) and Packard (1935) have determined the sensitivity of Drosophila eggs when x-rayed during different stages of embryonic development using hatchability as the criterion of radiosensitivity. Since in Habrobracon older embryos when x-rayed may hatch normally but may show deleterious effects after hatching, it seems that hatchability alone is not an adequate measure of radiosensitivity. Kelly (unpublished) showed that an x-ray dose of 30,000 r had no effect on the hatchability of older embryos of Habrobracon. Such embryos, however, would not have developed beyond the larval stage.

Although its significance is not known, the observation that nuclei of embryos arrested during cleavage are considerably larger following irradiation is in agreement with the reports of enlarged nuclei by Mottram (1933) for Colpidium, Jenson's rat sarcoma and bean roots, and by Harrington and Koza (1951) for grasshopper embryos.

These data on Habrobracon show that radiosensitivity cannot be correlated with genome number throughout the life cycle. It is difficult, therefore, to pose a single

generalization that will explain these diverse data from the standpoint of chromosomal injury. It may also be difficult to explain them on the basis of a quantitative difference of some chemical constituent. It is possible that there is no single "most-radiosensitive" mechanism, but that the relative sensitivities of the cellular materials change during development and that different mechanisms may be primarily involved at different stages.

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SUMMARY

1. Comparison of radiosensitivity of haploids and diploids of Habrobracon shows that the stage of development at which the animals are x-rayed is important in determining the relative sensitivity between these groups. When embryos are x-rayed during cleavage, haploids are more resistant than diploids; when embryos are x-rayed immediately after cleavage has been completed (blastema stage), haploids and diploids are equally radiosensitive.

2. Embryos x-rayed during cleavage or early blastema are deleteriously affected during the egg stage or not at all. Those that hatch complete post-embryonic development normally. Older embryos when x-rayed may hatch, but post-embryonic development is slowed down and many of the individuals are arrested in development as larvae. Hatchability, therefore, is not an adequate criterion of radiosensitivity for older embryos.

3. Embryos that are x-rayed during cleavage and fail to hatch are arrested in cleavage or in early blastema. The nuclei are arrested at interphase and become enlarged up to four times the diameter of untreated nuclei.

4. Since the differential radiosensitivity between haploids and diploids depends upon the stage of development at which they are irradiated, it is difficult to pose a single hypothesis that will account for these facts. It seems reasonable to consider that the relative sensitivities of the cellular materials change during development and that different mechanisms may be primarily involved at different stages.

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ADAPTATION TO CHEMICAL STIMULATION OF THE TARSAL RECEPTORS OF THE BLOWFLY[1].

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In the course of several decades of studies of chemoreception in insects the existence of some demonstrable form of adaptation was speculated upon by many workers. Some actually designed specific experiments to develop the idea. These experiments usually took the form of a comparison of a taste threshold measured by means of a descending series of concentrations with a threshold obtained by ascending presentation. Among the investigators who made the comparison, Marshall (1935) and Eger (1937) could demonstrate no difference; Verlaine (1927), Weis (1930) and Kunze (1933) believed that such differences existed, but their data in support of the belief are statistically poor; von Frisch (1934) could detect no change in threshold but did observe modifications of behavior which suggested that adaptation did occur; Minnich (1929) observed clear-cut differences in threshold. Both during and since that period extensive use was made of a convenient reflex of flies and butterflies, the proboscis response; but the data which the method yielded in the hands of the pioneer workers were contradictory and of questionable significance. Now, as a consequence of recent advances in the field, fuller advantage may be taken of this easily observed and decisive response to investigate profitably the phenomenon of adaptation and to reconcile the divergent older results.

When receptors located on the tarsi are stimulated by solutions of certain sugars, among them being sucrose, glucose and fructose, the insect responds by extending its proboscis and, when permitted, by drinking. Upon cessation of stimulation or initiation of stimulation by a wide variety of non-saccharide materials (*e.g.*, electrolytes and many organic compounds) the proboscis, if extended, is retracted. If, prior to stimulation by unacceptable compounds, the proboscis is already in the retracted position, there is no discernible response involving the mouthparts. In order to study the stimulating effect of unacceptable compounds it has been the custom to mix different amounts of the material in question with sugar solutions which are then presented to the insects. In this manner one is able to ascertain the minimum concentration of an unacceptable compound which will prevent a response to sugar. Thus, the two basic techniques employed in all experiments on contact chemoreception involve the determination of acceptance thresholds and of rejection thresholds.

Any of three standard operational procedures may be followed in obtaining threshold data: (1) each insect of a sample population of two hundred or more is stimulated by each concentration in turn presented in ascending order until a

[1] The work described in this paper was done under contract between the Medical Division, Chemical Corps, United States Army, and The Johns Hopkins University. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the author.

response is obtained; (2) each fly is stimulated by each concentration in turn presented in descending order until an appropriate response is elicited; (3) the insects are random-sampled, *i.e.*, a different group is tested at each concentration and note taken of the percentage of each class which responds. Median values for the threshold may be developed from any of these procedures by the proper mathematical treatment (see Dethier and Chadwick, 1948). As will be shown below, use of these procedures on a comparative basis affords excellent opportunities for investigating the phenomenon of adaptation.

I. Demonstration of Adaptation

Methods and results

Random Series. Several hundred one to three day old flies (*Phormia regina* Meigen) from a standard culture were mounted on sticks according to the method described by Dethier and Chadwick (1947). Just prior to testing, each fly was given water *ad libitum*. A series of sucrose solutions of doubling steps of concentration from 0.0005 *M* to 1 *M* was prepared. The flies were divided into as many groups of twenty each as there were concentrations of sucrose. Each group was tested at a different concentration. A test consisted of lowering the legs of each fly first into water until no proboscis extension was elicited and then placing the legs into the test solution for two seconds. The percentages of each group responding at each concentration were converted to probability units and plotted against the logarithms of the respective concentrations. The most probable value of the log concentration accepted by 50% of the flies was then determined according to the method of Bliss (1938).

Ascending Series. The same series of solutions as before was prepared. Each fly was tested as follows: (1) its legs were placed in water, and if a response was elicited, the animal was allowed to drink its fill; (2) when the response to water was negative, the fly was placed in 0.0005 *M* sucrose for two seconds; (3) if no response was forthcoming, the fly was transferred directly to the next higher concentration for two seconds and similarly on up the concentration series until a response was elicited. In no test was the proboscis ever allowed to touch the test solution. Once a fly had responded to a given concentration, it almost invariably responded to all higher concentrations. As soon as all flies had been tested the raw data were sampled in the following manner. A table with as many columns as there were concentrations was constructed. The specimens were now sampled, five at a time, in the order in which they had appeared for testing. For the first group of five the number accepting at the highest concentration was recorded in the table; for the next group, the number accepting at the next lower concentration; and so on in rotation until all of the flies used in the test had been recorded. The percentages accepting at each concentration were calculated from the totals in the columns and treated as above.

In an ascending series the responses of the population usually extended over ten doubling concentrations. Consequently, it was the custom to test a minimum of two hundred flies so that the *n* for each class was never less than twenty.

Descending Series. The method of running descending series differs from that of ascending series in the following respects. After being tested in water flies are presented first with a high concentration, *e.g.*, 1 *M*, known, as a result of informa-

tion derived from random series, to be above threshold. After two seconds each fly is then presented directly with the next lower concentration and so on down the series until the proboscis is withdrawn. When this occurs, the animal is retested against the starting concentration as a check against absolute refractoriness. If he continues down to the lowest concentration in the series with no sign of cessation of response, he is retested in water. Such a fly invariably drinks greedily, and the test is discarded. Not infrequently flies which are negative to water at the beginning of a run develop a desire for water in the course of the run. Data are accumulated and manipulated as already described. No significant difference in threshold is obtained if the feet are washed in water between successive concentrations provided the time consumed does not exceed a few seconds.

It is important to keep in mind the fact that descending series may be started at any supraliminal concentration.

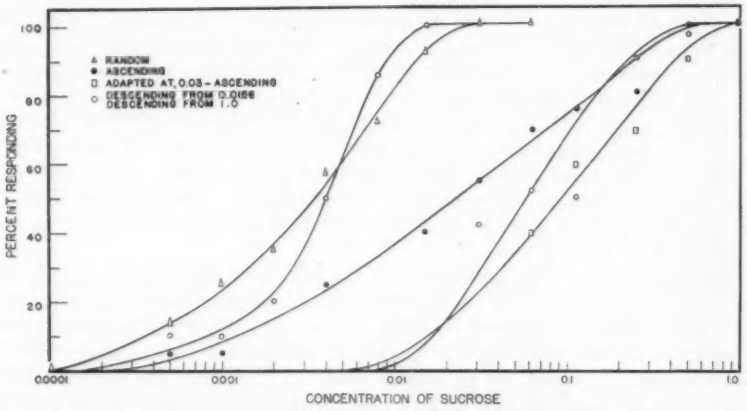

FIGURE 1. Change in the acceptance threshold of the blowfly to sucrose brought about by altering the order of presentation of different concentrations.

Adapted at One Concentration. It was feasible to start these series at any concentration, either subliminal or supraliminal. If, for example, a supraliminal concentration such as 0.03125 *M* was chosen as the starting point, each fly in turn was held with its feet in the solution until extension of the proboscis finally ceased. The animal was then run up the series in the usual manner until a fresh proboscis response was elicited. Again the data were random-sampled and treated as in the case of a normal ascending series. An alternative method was to random sample after adaptation. This procedure was carried out by dividing the flies into groups of twenty. Each fly in turn in group 1 was adapted, then tested at the next doubling concentration. The per cent responding was recorded. The members of group 2 were similarly adapted, then tested each in turn at the second highest doubling concentration and so on. If a subliminal concentration was chosen for adaptation, the feet were held in contact with the solution for ten seconds after which one of the procedures described above was followed.

Altogether, seven acceptance thresholds were determined by means of the techniques just described (see Section III): random sampled, ascending, descending from 1.0 *M*, descending from 0.0156 *M*, ascending after adaptation at 0.03125 *M*, random after adaptation at 0.0156 *M*, and random sampled after adaptation at 0.001 *M* (see Section III). Representative curves describing the distribution of these thresholds are seen in Figure 1. Statistical information relating to the median threshold values, standard errors, slopes and variance is given in Table I.

Discussion

It is clear from an inspection of Figure 1 that several different values for the acceptance threshold of sucrose by a given population of flies can be obtained and

TABLE I

Acceptance and rejection thresholds of Phormia regina as ascertained from different methods of presentation of stimuli

Method of presentation	Median acceptance threshold	Log molar concentration accepted by 50%	$a \pm$S.E.	$b \pm$S.E.	$\bar{x}$	No. of flies tested
Random	0.00356	−2.449±0.0332	4.983±0.0531	1.596±0.117	−2.555	120
Ascending	0.023	−1.632±0.0966	4.972±0.0992	1.028±0.123	−1.659	220
Descending						
From 0.0156 *M*	0.00334	−2.476±0.0598	5.016±0.136	2.285±0.310	−2.469	120
From 1.0 *M*	0.0474	−1.324±0.0739	5.450±0.119	1.834±0.268	−1.079	240
Adapted at 0.03125—then ascending	0.0953	−1.021±0.0999	5.510±0.146	1.810±0.381	−0.739	100
Adapted at 0.001 random 1 sec.	0.0116	−1.935±0.0477	5.188±0.104	2.242±0.271	−2.019	250
4 sec.	0.0156	−1.807±0.0472	4.901±0.176	3.753±0.630	−1.933	100
10 sec.	0.0168	−1.776±0.0050	4.891±0.0897	1.802±0.234	−1.782	250
30 sec.	0.0114	−1.942±0.0439	5.041±0.173	3.939±0.613	−1.932	80
Adapted at 0.0156 random 12 sec.	0.0458	−1.339±0.0472	5.058±0.158	3.356±0.464	−1.322	100
30 sec.	0.0676	−1.170±0.0505	4.996±0.146	2.895±0.392	−1.171	100
Ethanediol in fructose						
Random	8.128	0.910±0.018	5.281±0.137	7.647±1.059	0.946	120
Ascending	8.241	0.916±0.024	5.220±0.144	6.182±0.900	0.951	160
Fructose in ethanediol						
Random	0.142	−0.849±0.096	5.093±0.122	1.276±0.251	−0.777	120
Ascending	0.142	−0.848±0.066	5.380±0.150	2.364±0.414	−0.730	100

that the magnitude of these values depends upon the order in which stimuli are presented. It can also be shown (Section III) that time relations in the presentation of stimuli are of considerable importance. The data demonstrate that response to stimulation of the tarsi by sucrose is profoundly modified by previous stimulation. This effect of stimulation exhibits all of the characteristics of adaptation.

The curve describing the distribution of thresholds of a sample of flies which has been random-sampled can be taken as the baseline of sensitivity for this laboratory population at a given age and level of nutrition and water balance. To this baseline all other thresholds will be referred, because its median represents the lowest

concentration of sucrose which will elicit a response from 50% of the flies, a concentration which is effective if there has been no immediate previous stimulation. This basal value is 0.00356 *M*.

When successive concentrations of sucrose are presented in ascending order beginning at a concentration subliminal with respect to the random curve, the threshold is increased by a factor of approximately seven. In other words, individuals now fail to respond to solutions which are known to be capable of evoking a response if presented directly after water and in the absence of previous stimulation. Quite obviously the process of gradually increasing the stimulus by small increments from a subliminal value has caused a decrease of sensitivity to occur, so that a higher-than-normal concentration is finally required to trigger response. If this is so, it should be possible to effect a similar condition by prolonged exposure to a single selected concentration. This can be accomplished, as explained in the foregoing section, by placing the feet in a concentration which normally produces a response, waiting until the animal ceases responding, and then stimulating successively in an ascending series until the animal again responds. Similarly one can, after cessation of initial response, determine the final threshold randomly instead of successively. In any event the final threshold obtained depends upon the strength of the initial adapting solution. In the two cases tested the threshold is a concentration approximately three times that of the adapting concentration. Thus, it may be seen that the median value following adaptation at 0.0156 *M* is 0.04 *M* and following 0.03125 *M* it is 0.095 *M*. If adaptation is accomplished at a still higher concentration, the final threshold will be correspondingly higher.

Again, if the effects produced by previous stimulation partake of the nature of adaptation, one would logically expect that successive stimuli presented in decreasing order of concentration would yield a threshold value differing from those obtained with the foregoing methods. Further inspection of Figure 1 will show that this is indeed the case and that once again the value finally obtained depends upon the starting point of descent. When the initial stimulus is that which elicits approximately 100% response in the random series, *i.e.*, 0.0156 *M*, the final threshold does not differ greatly from the random series value. It is 0.00334 *M*. When the descent starts with 1 *M*, that which elicits 100% response from animals in an ascending series, the final threshold value (0.0474 *M*) is only slightly higher than the ascending value (0.023 *M*) but is many times higher than that of the other descending series. In other words, the final threshold value is determined by the point of departure.

These results naturally raise several critical questions. Can they be reconciled with the data of earlier workers? What are the respective contributions of the peripheral and the central nervous systems to the phenomenon? What is the nature of the process?

The failure of the majority of early workers to demonstrate clearly a difference in threshold with different techniques of presentation of stimuli, commonly ascending versus descending series, is due principally to the fact that such a long time interval was allowed to elapse between presentation of successive concentrations that recovery was essentially complete, and the effects of previous stimulation had been largely dissipated. Even under optimum conditions of presentation of stimuli the difference between ascending and descending threshold values is hardly demon-

strable if identical ranges of concentrations are employed in the test. Marshall (1935) and Eger (1937), working with honeybees and caterpillars, respectively, routinely allowed 15- to 20-minute intervals between stimuli; consequently, it is not surprising that no threshold differences were demonstrated. The differences that Weis (1930), Verlaine (1927) and Kunze (1933), working with *Pyrameis atalanta, Pieris rapae* and the honeybee, respectively, reported are not statistically significant. Again, failure to show pronounced differences may be laid to the selection of time intervals. Weis, for example, presented ascending series essentially as described in this study, but in her descending series interpolated a 15- to 20-minute rest period between successive stimuli. Moreover, as shown above, it is not to be expected that there would be a great difference between an ascending series and a descending one when the latter begins at the top concentration of the former. Minnich (1929), on the other hand, studying the responses of *Calliphora vomitoria,* was able to arrive at a clear-cut difference because he compared the threshold obtained from an ascending series run as in this study with a threshold obtained by an ascending series in which the intervals between stimuli were so long (15 minutes) that the series was tantamount to being random. A period of 15 minutes appeared ample for complete recovery.

Thus it may be concluded that the contradictions in the published reports are not real, that the data are actually in agreement, and that previous stimulation does indeed affect the sensitivity of the system at one or more points.

II. Site of Adaptation

Methods

In order to determine whether or not failure of response to threshold amounts of sucrose following previous stimulation results primarily from a change in sensitivity of the receptors themselves or to adaptation at some distance from the peripheral system, that is, at a site in the central nervous system, an attempt was made to devise a method for separating the two effects. This was accomplished in part by adapting one leg and then testing the sensitivity of the contralateral leg. Several techniques were tested before two were decided upon. The first consisted of mounting each fly with all legs fastened to the wax block except the prothoracic pair and then tying a piece of fine silk thread to one of the free legs. This leg could now be raised or lowered into the test solution at will. Each fly was run up an ascending series of solutions in the usual manner except that the fettered leg was held out of the solution. As soon as a threshold of response had been obtained with the free leg, the unexposed leg, together with the adapted leg, was placed in a solution which was less concentrated but which was known from random series to be above the threshold of sensitivity. Forty-three flies were tested by means of this exacting technique.

The second technique made use of a partitioned dish which contained 0.125 *M* sucrose in one compartment and water in the other. A fly with only the prothoracic legs free was made to straddle the partition. The labellum was removed to prevent drinking. A proboscis response followed as a consequence of stimulation of one leg by the sucrose. As soon as the fly had become completely adapted, *i.e.,* retracted the proboscis and ceased responding, the legs were thoroughly rinsed

in water, the fly was returned to the partitioned dish as before to insure that it was indeed adapted, it was again rinsed in water, and finally faced in the opposite direction and placed in the dish so that the adapted leg was now in the water compartment and the unexposed leg in the sucrose compartment. Thirty-six flies were tested.

Results and discussion

In every case in which hobbled flies were exposed successively to an ascending series of concentrations (first method), the fly afterwards failed to respond to a concentration below the ascending threshold when the unexposed contralateral leg was stimulated. A response could be elicited only by stimulating with a supraliminal concentration. Clearly, then, in this experiment the animal had become refractory in that it failed to respond even when a peripherally unadapted leg was stimulated. This behavior suggests that adaptation is central, but the possibility remains that the unnaturalness of the fettered condition rather than chemical adaptation may have been the limiting factor. However, the results were identical regardless of whether the free or the fettered leg was adapted.

On the other hand, when the divided dish was employed for testing, 66.6% of the flies which had been adapted on one leg responded vigorously when the contralateral leg was stimulated. Three major interpretations present themselves: (1) sucrose has not been washed from the stimulated leg completely, as a result of which the test with the contralateral leg actually amounted to bilateral stimulation; (2) adaptation is indeed peripheral; (3) adaptation occurs at a center in the CNS where there is no pooling of the contributions from both sides of the body.

With regard to the first possibility it can only be said that experience from many other sources would argue in favor of the rinsing being complete. If rinsing has not been adequate, the fact that addition of stimulation from the other leg provokes a response is an indication that the adapted leg is still contributing to the CNS and at least is not fully adapted; hence, the initial cessation of response results from CNS adaptation. On the other hand, if rinsing has been thorough, the return of response when the contralateral leg is stimulated indicates either that the first leg has been completely adapted or that the center into which it leads has been adapted. In either case stimulation of the fresh leg would activate a different site in the CNS and the animal would respond. The possibility of solving this problem appears remote. It must be added that not one of the 33.3% which failed to respond would respond (after adaptation) when both legs were placed simultaneously in 0.125 *M* sucrose. A greater concentration did provoke a response. Clearly these cases are indicative of a change in the central nervous system.

While it is surely unlikely that there is no adaptation in the receptors themselves, the more acceptable conclusion, all facts considered, is that the process which is being measured represents predominantly a state which has been brought about at some level in the central nervous system. If this be so, the condition may be analogous to the non-sensory adaptation in the olfactory system of man (Adrian, 1950) or to the phenomenon of inattention in general. It is indeed unfortunate that the tarsal system has not yet proved vulnerable to attack by bioelectrical techniques.

III. Time Course of Adaptation

Methods

Three different experiments were designed as a means of supplying information necessary for the plotting of adaptation curves. The first two conform more or less closely to the classical method of securing data for adaptation curves. Flies were adapted for varying periods of time to a selected concentration and the threshold measured after each period of adaptation. The experiment was repeated for several adapting concentrations. In the first method the adapting concentration was above the threshold of response (supraliminal). The actual method of procedure involved placing each fly of a sample of twenty in the adapting solution until the proboscis was retracted. This event was taken to mark zero time. The fly remained in contact with the solution for one second after zero time, after which it was exposed to a higher concentration for two seconds and the presence or absence of a response noted. Each remaining fly of the sample was treated similarly. Now another sample of twenty flies was placed one at a time in the same adapting solution also for one second and then tested at higher solution number two; a third sample was tested at higher solution number three and so on until five different samples of flies had each been adapted at the same concentration for an identical period of time and then tested for response at five different high concentrations. The per cent responding at each concentration was then calculated and from these values was determined the acceptance threshold characteristic of flies which had been adapted at a given solution for one second. The entire procedure was repeated at the same adapting solution for a period of two seconds, three seconds, and so on until a sufficient number of data had been obtained to plot a curve for that particular adapting concentration. The experiment was repeated in its entirety for another adapting concentration.

Method number two differed from the foregoing in one respect only. The adapting solutions were subliminal. Since there was no proboscis response to these adapting solutions, zero time was taken as the time at which the tarsi first came into contact with the solution.

Method number three consisted simply of ascertaining the time required for complete adaptation at each of several different concentrations. Data were obtained for each concentration by placing the feet of a fly in the solution and recording the interval of time which elapsed between extension of the proboscis, which occurred almost immediately after the feet touched the solution, and retraction. One hundred flies were tested at each concentration. It should be emphasized that the mouthparts were at no time in contact with the solutions.

The results of these three experiments are summarized in Table I. Data obtained by method number three are plotted in Figure 2.

Discussion

The fact that the rise in threshold following adaptation to supraliminal concentrations (method one) is independent of the duration of stimulation, once the proboscis has been retracted, and is constant, suggests that the change in sensitivity has attained completion at the time of retraction. The magnitude of the completed change varies as the adapting concentration, as a comparison of thresholds after continued exposure to different adapting concentrations has shown (Section I),

but in each case it is complete. It follows from this that the development of adaptation must be studied at subliminal concentrations. When this is done (method 2) at 0.001 *M* for example, there are indications of an increase in threshold as the duration of continued stimulation increases. Thus, at zero time (no previous exposure) the threshold is 0.00356 *M;* at one second it is 0.0116 *M,* at 10 seconds

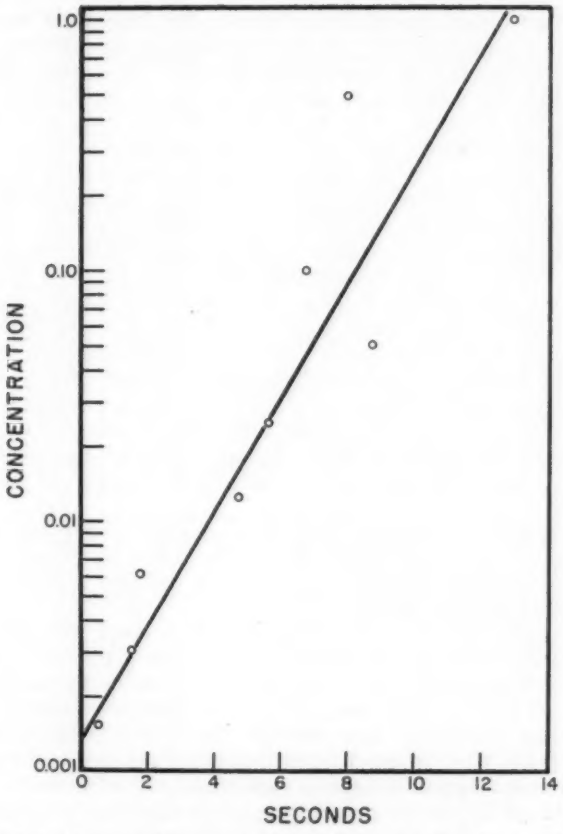

FIGURE 2. The relation between the concentration of sucrose employed as a stimulus and the time required for the cessation of response.

it is 0.0168 *M*. No further increase can be demonstrated up to and including 30 seconds. From these data it would appear that adaptation of the type being measured at 0.001 *M* is complete after one second. Increases from one to ten seconds, while progressing in the correct direction, are not statistically significant. Since it is manifestly impossible with the preparation at hand to investigate sensitivity changes occurring in fractions of a second, further indications as to the shape

of the curve of adaptation cannot be obtained. On the other hand, it is possible experimentally to ascertain the time necessary for complete adaptation at different concentrations (method 3).

As an examination of Figure 2 shows, the time for complete adaptation as measured by time to retraction of the proboscis increases as the logarithm of adapting concentration. The increase is from about 1.5 seconds at 0.003125 *M* to about 13 seconds at 1.0 *M*. These data are in agreement with those from methods one and two in that method one indicates adaptation to be complete when the proboscis is retracted and method two indicates that at 0.001 *M* adaptation is complete after one second or less. A less complete set of data obtained with fructose in ethylene glycol is in substantial agreement and shows an increase in adaptation time from about 4 seconds at 0.006 *M* to 7 seconds at 1.0 *M*.

Even though it has not been possible to construct adaptation curves for *Phormia*, the fact that a correlation with adapting concentration can be demonstrated permits one to compare certain aspects of the phenomenon in *Phormia* and man. Hahn (1933) and Hahn, Kuckulies and Bissar (1940) have measured the course of adaptation of a variety of substances at different concentrations. The forms of the curves differ considerably, but two facts are worthy of mention: adaptation is complete in anywhere from 10 to 30 seconds; the time required for total adaptation increases as the concentration is increased. The situation in *Phormia* is remarkably similar. Adaptation times vary from one to 13 seconds; the total time required increases as the concentration is increased. Over the range of lower concentrations, 0.003125 *M* to 0.05 *M*, the time required is proportional to the logarithm of molar concentration. From 0.05 *M* on there is no significant change in time with increasing concentration.

Continuous stimulation at constant intensity for long periods of time results in bursts of activity. For example, a fly with its feet exposed to 0.05 *M* sucrose will continue to extend its proboscis for a period of 13 seconds on the average. Thereupon, the proboscis is retracted. After a varying period of time extension recommences; then it again ceases. Such rhythmic activity has been observed over periods of continuous stimulation lasting as long as three hours. Thus far no correlation between frequency of activity cycles and concentration has been observed.

IV. Simultaneous Stimulation by Mixtures of Solutions

No experiments were designed specifically to demonstrate the presence or absence of adaptation to compounds other than sugars, but work done in another connection throws some light on this problem.

Thresholds measured by the random technique and in ascending series were compared for each of two different mixtures, fructose in glycol, and glycol in fructose. In other words, determinations were made of the acceptance threshold of fructose in glycol and of the rejection threshold of glycol in 0.05 *M* fructose. The pertinent data are summarized in Table I.

In the case of each mixture there is no difference in the threshold value obtained by the random and the ascending methods. The most logical explanation of this fact is that adaptation occurs to each component of the mixture and that the processes balance each other. Otherwise, were there to be adaptation to fructose alone, the acceptance threshold should be higher in the ascending series.

The acceptance threshold would be lower in the ascending series if there were adaptation to the glycol alone. Similarly, were there adaptation to fructose alone, the rejection threshold to glycol would be lower in the ascending series. Since adaptation to fructose in glycol most certainly does occur, as was shown in Section III, it must be concluded that in mixtures of the sort described either adaptation to the two components progresses apace or that cross adaptation occurs. This last is highly unlikely.

Conclusion

The universality of the phenomenon of adaptation, in nervous tissue especially, would have led one to infer that tarsal chemoreceptors in blowflies could hardly act otherwise. The chief problem heretofore had been to demonstrate the existence of the process. Once demonstrated the problem remained of separating the sensory and central components. Unfortunately, realization of this hope has been but partial. The present data indicate, as would also have been expected, that the process as measured by behavior represents a combination of both components. Indications are that the central process proceeds to completion more rapidly and that it is the one which is manifested in the majority of experiments.

A more ambitious hope lay in the expectation that knowledge of the time course characteristics of the process might lead to clues to the nature of the process of chemoreception. This has so far been a barren hope, not only in the field of insect physiology but in the study of the human sense of taste as well. More fruitful comparisons do exist. The times required for total adaptation in the two species are of the same order of magnitude although in man the process which is measured is almost certainly peripheral; the time to total adaptation increases as the adapting concentration increases.

The quickness of adaptation would tend to nullify the effectiveness of contact repellents; however, the corresponding speed of recovery counteracts this. Consequently, the existence of sensory adaptation to chemicals does not appear to be a factor which causes any appreciable reduction in the response of insects to repellents. It would thus appear that the process of adaptation plays no great part in the loss of repellency with time.

Certain characteristics of the response illustrate very beautifully some of the concepts of behavior recently reviewed by Lorenz (1950). In addition to exhibiting the type of adaptation described herein, it parallels in its action many other aspects of behavior seen in vertebrate animals. For example, under continuous stimulation of long duration the fly responds with bursts of activity; it shows an inertia of reaction in that removal of the stimulus, even when followed by rigorous cleansing in water, does not result in immediate cessation of response. On the other hand, proboscis extension *in vacuo*, that is, in the absence of stimulation after prolonged inactivity, has not been observed. The response is fully deserving of more intensive investigation because it offers unusual opportunities for establishing a neural basis for many commonly observed patterns of behavior.

Summary

1. When receptors located in the tarsi of the blowfly *Phormia regina* are stimulated by solutions of sucrose, the insect responds by extending its proboscis.

2. The lowest concentration which would elicit a response from 50% of the population studied was taken as the acceptance threshold. The threshold value obtained depended upon the order in which different concentrations were offered for testing.

3. Adaptation was demonstrated by comparing threshold values obtained (1) by offering solutions in ascending series of concentrations, (2) in descending series, (3) by a random technique, (4) following exposure of the tarsi to subliminal concentrations in one case and supraliminal in the other. The process of adaptation which was measured represented predominantly a state which had been brought about at some level in the central nervous system.

4. The time required for adaptation increased as the logarithm of concentration. For sucrose it varied from one to 13 seconds. Stimulation by mixtures of glycol (unacceptable) and fructose (acceptable) resulted in adaptation to each component.

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A STUDY OF RADIOPHOSPHATE UPTAKE IN PARAMECIUM MULTIMICRONUCLEATUM

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It was our original intent to find out if it is possible, by the use of radioactive elements, to tag microorganisms such as Paramecium, in order that an adequate evaluation of their place in food chains could more accurately be determined. It is possible under certain conditions to render paramecia sufficiently radioactive with inorganic radiophosphate that single animals will give a definite positive count over background. Thus it should be quite possible by means of simple predation experiments to calculate the utilization of microorganisms in specified situations. Coffin *et al.* (1949) and Hayes and Coffin (1951), by adding P^{32} to small lakes, have established by this relatively simple means that phosphate distribution among members of the community was much more rapid than previously supposed and that there was an extensive exchange of elements between organisms. Phosphate turnover was greatest among the algae. Another interesting fact revealed by their results was that certain plants concentrate P^{32} up to 40,000 times the concentration in surrounding water.

It is important to know to what extent aquatic organisms concentrate ions in their bodies from their environment because of the implications in sewage and radioactive waste disposal problems.

Methods

The organisms used in these experiments were derived from the cultures used by Professor W. H. Johnson of Wabash College in his studies on the growth of *Paramecium multimicronucleatum* Powers and Mitchell in bacteria-free culture. All cultures were grown as described by Evans (1944). Stock as well as experimental cultures were fed on yeast and grown at room temperature (24–27° C.). At the end of 10 days, the population of paramecia reached a peak of about 450 individuals per ml. During the latter part of the logarithmic growth phase, the P^{32} was introduced as NaH_2PO_4 in weak acid solution. No carrier was added and no buffer was needed since the radioactive solution was diluted from one part in 250 of Osterhout's culture medium to one part in 2500 of Osterhout's. The Osterhout's was buffered with $M/20$ phosphate and adjusted to a pH of 7.0 with $M/10$ NaOH. The pH was measured electrochemically with a Beckman pH meter.

Several cultures for the experiments were grown; the amount of P^{32} added varied from 0.1 to 10.0 microcuries per ml. Organisms were withdrawn at intervals and washed as free as possible of the external radioactive phosphate either by dilution following centrifugations or by pipetting. Supernatant fluid from the last wash was always checked for radioactivity. In some cases, P^{32} was added to

unfed cultures, but in all other respects the organisms were treated as described above.

The washed organisms in counted numbers were placed in metal planchets with a minimum of fluid and desiccated; single animals were transferred with $\frac{1}{500}$ ml. of fluid, 10 animals with $\frac{1}{50}$ ml., and 100 animals with $\frac{1}{20}$ ml.

In order to determine if the washing was adequate, one-ml. samples of supernatant fluid from all of the successive washings were tested. Similar tests were carried out on solutions through which paramecia were transferred during washing with pipettes. It was found that no practical reduction in residual radioactivity could be effected after the fifth centrifugation. Even after 12 centrifugations, there remained some radioactivity in the supernatant fluid. Five washings brought the count down to about 10 counts per minute per ml. Kamen and Spiegelman (1948) noted that rapid centrifugation of Rhodospirillum caused considerable leakage of phosphate ions from the cells. This was also true but less so for Chlorella. They state that yeast gives up negligible amounts of P^{32}. Likewise, Moraczewski and Kelsey (1948) found that *Trypanosoma equiperdum* gave up phosphorus continuously especially as the activity of the animals decreased. They associated this with increased permeability as death of the cells approached. However, Labaw *et al.* (1950) state that after inorganic phosphate has been incorporated into the nucleic acid molecule in *Escherichia coli,* there is no interchange of the phosphorus either by metabolic exchange or death.

A certain amount of turnover is normal, but centrifugation seemed to enhance the loss of P^{32} from the cell. It was calculated that the amount of radioactivity in the medium carried over with the paramecia from the last wash to the planchets for desiccation was negligible.

Washing away the radioactive external medium by transferring with micropipettes was more satisfactory when only a few animals were desired, but this method was too slow for washing large numbers of organisms.

In a typical experiment, hundreds of paramecia were drawn out of a radioactive culture in one ml. of medium and deposited in a 15 ml. centrifuge tube. To this was added 13 ml. of fresh balanced salt solution (Osterhout's). The paramecia were centrifuged at 34 gravities for 10–12 seconds. This was sufficient to bring all normal paramecia to the bottom, but starved paramecia required about 300 gravities for 30 seconds. A glass plunger was inserted, one ml. of the medium removed for testing, and the supernatant poured off. Fresh balanced salt solution was again added and the process repeated as many times as was necessary.

Radioactivity was determined by means of a standard 64-scale Geiger counter with a Tracerlab mica window tube 1.9 mg./cm.^{2} in window thickness. The specimens were centered in planchets and counted at a distance of 5 cm. from the center of the tube. Each sample was counted for five minutes. Those with especially high levels of radioactivity were counted for one minute. All counts were corrected for decay.

Results

The presence of the radioactive phosphorus in the paramecia cultures did not appear to affect the course of the cultural history. The organisms remained the same size, their behavior was unmodified and the reproductive rate, though not measured exactly, appeared to parallel control cultures.

In the initial experiment, 0.5 microcurie of P^{32} per ml. was added to a culture of paramecia. The organisms were dividing at a maximum rate. The culture was one week old; there were approximately 350 paramecia per ml. Twenty-four hours after introduction of the P^{32}, paramecia were removed and prepared for radioactive determinations either singly or in groups of ten. While the single paramecia always registered a positive count over background it was low, averaging 3.8 counts per minute. The groups of 10 paramecia per dish showed an increase by a factor of 10 over the singles; this confirms the reliability of the individual counts.

Six days later, the radioactivity of the paramecia was again measured. The activity had increased slightly, being now 4.5 counts per minute per animal. Again the dishes containing 10 animals each showed a very close correlation with the 10 separates.

It is to be noted that the paramecia were feeding and possibly most of the intake of phosphate was through the food vacuole system, either in solution or in the food organisms.

Several new cultures, each containing thousands of thoroughly washed paramecia, were established. These received no food. Radiophosphorus was added and radioactive determinations were made at intervals (after 20 hours, 3 days, 5 days, 9 days and 11 days). The concentrations tested were 0.1, 0.2, 0.5, 0.8 and 1.0 microcuries per ml. Three cultures were set up at each of these concentrations.

The results of these experiments showed that paramecia which have no available food take in very little P^{32}. In fact, groups of 100 paramecia averaged only 8.3 counts per minute for an average of 0.08 counts per animal per minute. There was no difference in uptake between the weakest and greatest concentrations of radioactive substance. Time had little effect; the organisms were about as radioactive after 20 hours as at the conclusion of the experiment.

On the eleventh day, yeast was added to two of the cultures containing 1.0 microcurie of P^{32} per ml. The population soon began to increase. Twenty hours later, radioactivity counts nearly equaled those of paramecia in the initial experiment.

Inasmuch as many microorganisms, especially green forms, may concentrate inorganic ions inside the cell, it might be revealing to render old cultures of paramecia radioactive. Andresen *et al.* (1950), using C^{14}, were able to concentrate this element in *Stentor polymorphus* containing symbiotic Chlorella. The Stentor individuals finally yielded 283 counts per minute. Old cultures of paramecia become rust-colored and sometimes green due to the presence of bacteria and Chlorella which the paramecia ingest.

The old cultures chosen were three months old and the paramecia were very numerous and healthy. Four old cultures were selected; to two were added 0.1 microcurie of P^{32} per ml., and to the other two cultures were added 2.0 microcuries per ml. Four new cultures were started and run parallel with the old cultures.

The paramecia of the new and old cultures containing 0.1 microcurie of P^{32} per ml. showed a significant difference in radioactivity. Paramecia from old cultures showed consistently twice the radioactivity as those from new cultures. There was an even greater difference between paramecia taken from new and old cultures containing 2.0 microcuries of P^{32} per ml. Paramecia from the old cultures were five times as radioactive as were those from the new cultures. Apparently much

of their phosphate was acquired as food. The results also show that the greater the concentration of radioactive phosphate, the greater the amount of radioactivity in the paramecia. It should be pointed out that paramecia of old cultures are primarily non-dividing.

After absorption, is the phosphate tightly held in the cell or is it readily released? This question was partially answered by a series of tests. A few hundred radioactive paramecia were washed through 10 centrifugations. These were left in one ml. of the last wash. Five such tests were run. One tenth ml. of solution was removed at intervals, and the radioactivity tested. Table I shows the results.

TABLE I

Loss of P^{32} from Paramecium determined by radioactive measurement of 0.1 ml. samples of medium

Time in hours after washing paramecia in fresh medium	Counts per minute of 0.1 ml. of medium
0	1.5
24	64
66	95
116	154
168	167
192	170

The paramecia rapidly release the phosphate. Powers (1947), rendering *Paramecium aurelia* radioactive by feeding them radioactive *Aerobacter aerogenes*, noted a loss of more than one-half of the total phosphorus after 20 hours from the time the cells were separated from the source of radiophosphorus.

DISCUSSION

Paramecium multimicronucleatum can be made sufficiently radioactive with P^{32} to serve in predation experiments. However, in view of the fact that paramecia give up large amounts of radioactive phosphate within a few days, the duration of such experiments must be limited. Perhaps other radioactive substances will be found to remain longer or even indefinitely in the body of Paramecium. One interspecies experimental population, using radioactive paramecia as prey and *Didinium nasutum* as predator, has shown that the radioactivity of the prey is taken over almost if not entirely by the predator, and that the radioactivity of a dividing Didinium seems to be about equally distributed between the two daughter cells.

In natural populations, the amount of food present for Paramecium would be a deciding factor in uptake of P^{32}. Unfed paramecia seem unable to concentrate inorganic phosphate. Phosphorus is required for metabolism by microorganisms (Elliott and Hunter, 1951; Sullivan, 1950; Weisz, 1949), and the experiments reported herein indicate that most of the phosphate obtained by *Paramecium multimicronucleatum* is acquired in its food. Probably small amounts are absorbed directly and some adsorbed on the surface. Saprophytic and autotrophic forms seem to be able to absorb large amounts of phosphate directly.

Using Popoff's (1909) equation for determining volume of *P. caudatum*, it was calculated that paramecia which are feeding have roughly 20 times more radioactivity than the surrounding medium, while unfed paramecia have less than one-half the radioactivity. Mazia and Hirshfield (1950) measured the uptake of in-

organic P^{32} by Amoeba in the absence of food, and they found that the concentration of phosphate in the cell was greater than outside by a factor of at least 50. It is probable that, as in *P. aurelia* (Powers, 1947), the phosphate is present in *P. multimicronucleatum* as organic phosphate.

Summary

1. In a medium containing inorganic radioactive phosphorus, *Paramecium multimicronucleatum* become sufficiently radioactive for use in quantitative predation experiments.

2. It is possible to measure the P^{32} uptake of a single individual.

3. When food is absent, Paramecium does not take in inorganic phosphate in solution. The phosphate is acquired in measurable amounts in its food.

4. The phosphate taken in is rapidly lost from the animals, probably as a result of an organic turnover.

5. Paramecia in old cultures, especially cultures containing Chlorella, become more radioactive than those in other cultures probably because the Chlorella which are ingested by the paramecia, absorb much phosphate, and also because old cultures of paramecia are primarily non-dividing.

6. The greater the initial concentration of P^{32} in solution, the more radioactive the paramecia become.

7. When washed by centrifugation, there is a leakage of P^{32} from the organisms over and above the normal turnover of phosphate.

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FURTHER NOTES ON THE TURBELLARIAN FAUNA OF THE ATLANTIC COAST OF THE UNITED STATES

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Since my previous publications on the larger Turbellaria of the Atlantic coast of the United States (Hyman, 1939a, 1940, 1944), very little in the way of new material has come to hand. Undoubtedly much remains to be done on the smaller Turbellaria of the region in question—acoels, rhabdocoels, and alloeocoels—but one may assume that our knowledge of the larger forms, especially polyclads, is practically complete. Florida remains the only region from which additional species may be expected. The present paper aims to put on record some new material and information and to call attention to necessary nomenclatorial changes. As the classificatory position and familial and generic definitions for the following forms were given in the publications cited it appears unnecessary to repeat them here.

Order Polycladida

Family Plehniidae

Plehnia ellipsoides (Girard), 1854, new combination

Discocelides ellipsoides Hyman, 1940

In connection with the finding of a new species of *Plehnia* on the Californian coast I am giving some discussion of the family Plehniidae and its genera that leads me to the desirability of transferring *Leptoplana ellipsoides* Girard, which I determined some years ago as a member of the Plehniidae, to the genus *Plehnia*. I now feel that it fits better in this genus than in *Discocelides* to which I previously assigned it.

Family Cryptocelidae

Phaenocelis purpurea (Schmarda), 1859

Comprostatum insularis Hyman, 1944

At the time of my description of this cryptocelid from the Florida keys, I considered it a new species representative of a new genus for which I created the name *Comprostatum*. Dr. Stephen Prudhoe of the British Museum (Natural History) kindly called my attention to the genus *Phaenocelis* Stummer-Traunfels, 1933. Stummer-Traunfels in 1933 re-investigated the types of Schmarda (1859) and determined that Schmarda's species *Leptoplana purpurea* from Jamaica is in fact a cryptocelid to which he gave the generic name *Phaenocelis*. There can be no doubt that my genus *Comprostatum* is identical with *Phaenocelis* and after some hesitation I am also now of the opinion that my species is identical with that of Schmarda. Hence the name *Comprostatum insularis* becomes a synonym of *Phaenocelis purpurea*.

Family Leptoplanidae

Zygantroplana angusta (Verrill), 1892, new combination

Stylochoplana angusta Hyman, 1939

In re-investigating from the original material *Leptoplana angusta* Verrill, 1892, now known to be native to the southern part of the Atlantic coast, I tentatively placed it in the genus *Stylochoplana* while recognizing some lack of conformity with this genus. Dr. Ernesto Marcus of the University of São Paulo, Brazil, kindly called my attention to the genus *Zygantroplana* Laidlaw, 1906, which I had unfortunately overlooked. Verrill's species fits perfectly into this genus and hence its name becomes *Zygantroplana angusta*. It is interesting to note that a species of *Zygantroplana*, so similar to *angusta* that it should probably be regarded as a geographic variant of the latter, is very common on the Brazilian coast near São Paulo (Correa, 1949). This would seem to be the first instance of a similarity in the Turbellarian fauna between Florida and Brazil.

Family Pseudoceridae

The genus Thysanozoon in Florida

The genus *Thysanozoon* is probably the easiest of all polyclad genera to recognize since the clothing of the dorsal surface with numerous, closely placed, conspicuous papillae is, by definition, diagnostic of the genus. By the same token, the species of the genus are particularly difficult to differentiate because of a general similarity of internal structure. Color becomes of considerable importance in differentiating the species and this is lost in preserved specimens. In 1851 Girard described under the name *Thysanozoon nigrum* a single specimen of a black *Thysanozoon* that he had taken on the eastern coast of Florida. Verrill (1901) recorded what appears to be Girard's species from Bermuda and during a stay at Bermuda in 1935, I also took two specimens alive (Hyman, 1939b). The Bermuda specimens were a rich velvety black throughout the dorsal surface with some small white flecks. Sections of the copulatory apparatus showed distinctive characters. *T. nigrum* was not again taken on the Florida coast, however, until 1950 when Dr. Harold Humm, in charge of the Florida State University marine station at Alligator Harbor near Tallahassee, kindly sent me a living *Thysanozoon* from that locality, collected February 4. This was 40 mm. long, of an oblong shape when crawling extended, and of a general dark grayish black, with white flecks on the margins and papillae. The animal was fortunately sexually mature and sections showed it to be identical with the Bermuda specimens. *Thysanozoon nigrum* therefore occurs both on the Florida coast and at Bermuda. It is a valid species distinguishable by the uniform black color of both dorsal surface and papillae, by the paired male apparatuses, by certain details of the male apparatus described in my Bermuda article, and by the presence of an intestinal branch in each papilla. Stummer-Traunfels (1895) in a review of the genus *Thysanozoon* pointed out that part of the species have but one male apparatus and that the occurrence of an intestinal branch in each papilla was known only for the type species, *T. brochii*. Marcus (1949) in reviewing the valid species of the genus records 9 with single and 15 with double male apparatus but does not mention the question of the pres-

ence of an intestinal branch in the papillae except with regard to his new species in which such branches are wanting.

There is another Floridan species of *Thysanozoon* besides *nigrum*. Pearse (1938) records taking no less than 29 specimens of a *Thysanozoon* in Crooked Island Sound and St. Joseph's Bay, localities in the same general part of Florida as Alligator Harbor. Pearse identified these specimens as *T. brochii* but described only the color which varied from cream to brown and purple with brown and purplish papillae. A whole mount of one of these specimens has come into my possession but as it appears immature (length 20 mm.) has not been sectioned. The extension of an intestinal branch into each papilla is clearly seen upon the whole mount, which thus does resemble *brochii* in this respect. What seems to be a specimen of the same species has been received for identification from the United States National Museum. This was collected by F. M. Bayer, January 26, 1951, under stones along the County Causeway, Biscayne Bay, Florida, which lies along the southern end of the eastern coast. This specimen also unfortunately proved sexually immature but in the cleared worm the intestinal network sending a branch into each papilla was evident. The specimen was accompanied by a color description which stated that the animal was purplish maroon with brown papillae and some white spots along the edge and on the papillae. There would seem little doubt that this specimen is identical with those of Pearse but whether they can be regarded as *T. brochii* remains uncertain.

Still another preserved specimen was received from the Florida State University, collected in November, 1938, at Clearwater, Florida, located at about the middle of the western coast of the peninsula. This specimen had been bleached white by the preservative and was also immature so that it proved of no value. Whether the intestinal branches extend into the papillae could not be determined.[1]

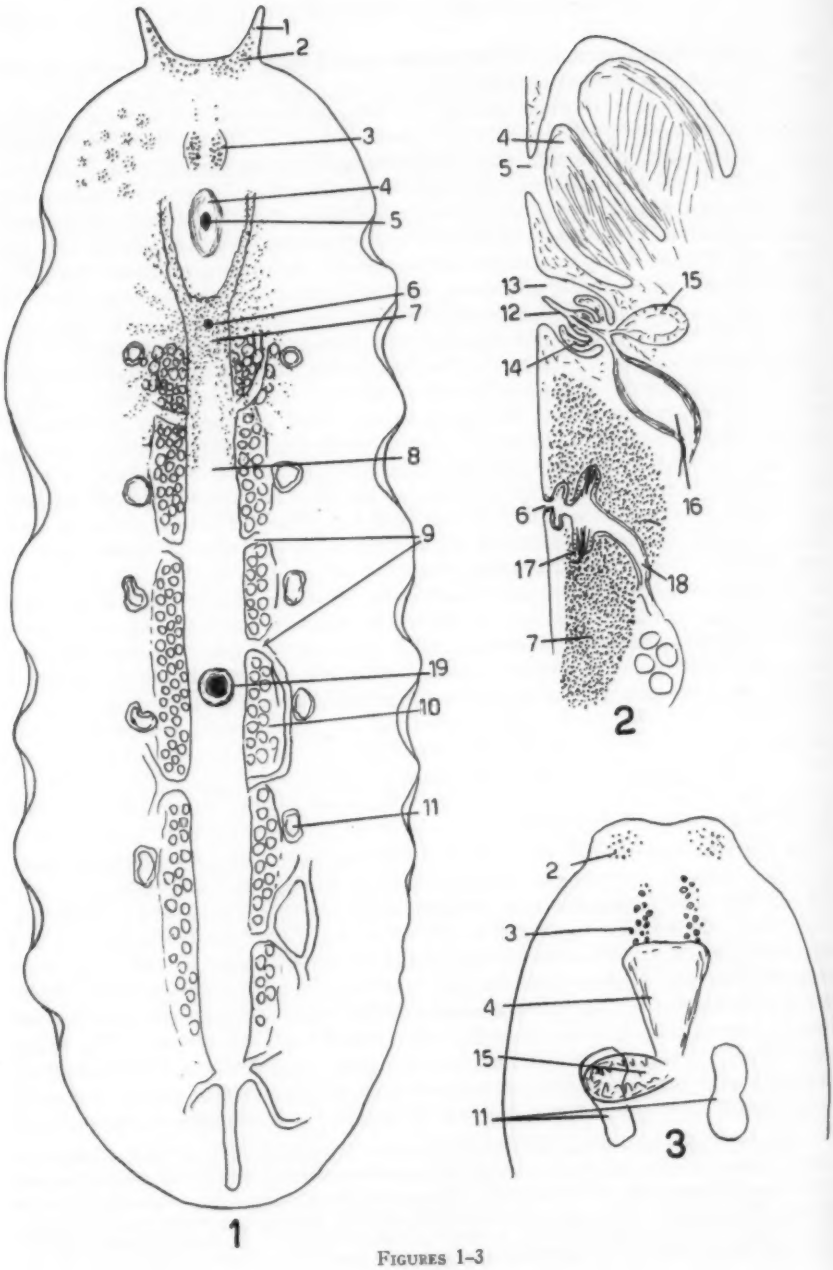
Family Euryleptidae

Prostheceraeus maculosus (Verrill), 1892, new combination

Eurylepta maculosa Verrill, 1892

In previous comments on this species I remarked that some good specimens were necessary before its systematic position could be determined with certainty. Apparently the species had not been refound since Verrill's publication. Therefore I was very pleased to be informed by Dr. Chauncey Goodchild of the staff of Invertebrate Zoology at Woods Hole that he had found the species in some abundance on wharf pilings at Lagoon Pond Bridge, Martha's Vineyard. On August 2, 1949, Dr. Goodchild kindly brought me a number of live specimens from that locality and also very generously took the trouble to fix a number of them for me. The living animal (Fig. 1) is about 10 mm. long when moving extended, and of an oblong or elongated oval shape with two pointed tentacles at the anterior margin. It sails along rapidly over the seaweeds by undulating the body margins after the usual

[1] Since the above was sent to press there has been received from the U. S. National Museum a vial of polyclads collected in the Biscayne Bay region of Florida by F. M. Bayer. This vial contains another specimen of *Thysanozoon nigrum*, and two specimens of *Pseudoceros crozieri* Hyman, 1939, hitherto known only from Bermuda and thus furnishing a second case of a polyclad common to Bermuda and Florida; further two specimens of the *Acerotisa* mentioned above, clearly an undescribed species, and an undescribed leptoplanid.



FIGURES 1–3

manner of cotylean polyclads. The color is light tan dotted with dark brown spots. Numerous eyes are present along each tentacle and on the body margin between the tentacle bases; there is also a pair of cerebral eye clusters over the brain region. Examination of pressed live specimens showed at once that the intestinal branches are anastomosed into a network and this puts the species in the genus *Prosthecereaeus*. Several specimens were sectioned and these also show the characters of *Prosthecereaeus*. Some details from these sections have been added to Figure 1. The main intestine forks anteriorly around the pharynx and behind this gives off irregularly several branches on each side that anastomose into a network. Along either side of the main intestine is seen an elongated tubular uterus stuffed with eggs and this gives off laterally uterine vesicles. In a specimen sectioned frontally five of these vesicles were seen on each side alternating with the main intestinal branches as characteristic of the genus. The sucker is situated at about the body middle. A sagittal view of the male and female copulatory apparatus is shown in Figure 2 but offers nothing particularly distinctive. It is unfortunate for systematic purposes that the copulatory apparatuses are very similar throughout the Cotylea, a fact that adds to the difficulties of specific identification in this group. A whole mount of the species has been deposited in the American Museum of Natural History.

The genus *Acerotisa* on the Atlantic coast

In 1940 I described *Acerotisa baiae* from a single specimen collected by Pearse in St. Joseph's Bay, Florida. I have here to record the unexpected finding of a specimen of this species by Dr. Chauncey Goodchild at Lagoon Pond Bridge, Martha's Vineyard, August 13, 1948. Alive the specimen was egg-shaped with pointed anterior end and of a translucent white color with the dorsal surface dotted with cream-colored dots. One may also expect to find from Woods Hole southward in the floating *Sargassum* the minute species *Acerotisa notulata* (Bosc) 1801, of which I gave a description in my article on Sargassum Turbellaria (Hyman, 1939b). Still another *Acerotisa* species occurs on the Atlantic coast of the United States. I am again indebted to Dr. Harold Humm for sending a living specimen of an *Acerotisa* obviously different from the foregoing species. This was collected April 16, 1951, at Alligator Harbor, Florida. Unfortunately it arrived partly disintegrated; what remained is shown in Figure 3. The numerous eyes in both the marginal and cerebral clusters clearly differentiate the animal from any other *Acerotisa* species known from the Atlantic coast or Gulf of Mexico. The animal was white peppered with dark dots. The animal bears a striking resemblance to *Acerotisa piscatoria* Marcus 1947, collected near São Paulo, Brazil, but the identity cannot be established without further material.

FIGURE 1. *Prosthecereaeus maculosus*, from life, some details added from sections.

FIGURE 2. Median sagittal section of pharyngeal region of *Prosthecereaeus maculosus*, showing male and female copulatory apparatus.

FIGURE 3. Anterior part of an undescribed species of *Acerotisa* from the Gulf coast of Florida, from life.

1, tentacles; 2, tentacular eyes; 3, cerebral eyes; 4, pharynx; 5, mouth; 6, female gonopore; 7, cement glands around female canal; 8, main intestine; 9, intestinal branches; 10, uterus; 11, uterine vesicles; 12, penis papilla; 13, male gonopore; 14, penis sheath; 15, prostatic vesicle; 16, seminal vesicle; 17, cement pouch of female canal; 18, vagina; 19, sucker.

Order Tricladida

Note on *Probursa veneris*

In 1944, I described the interesting marine triclad, *Probursa veneris*, found sheltering in empty *Venus* shells on a sand bar near the western end of the Atlantic shore of Long Island. It was therefore very surprising to receive a number of live specimens of this worm from Dr. Harold Humm, who had found them in Bogue Sound, near Beaufort, North Carolina, June 20, 1946, on the fronds of a red alga, *Mitophyllum medium*. Not only the adults but also many young and spherical orange capsules were present on the alga. Alive the worms were translucent white, very active and restless, swimming by a twittering motion, and crawling in leechlike fashion by alternately attaching the body ends, that must be provided with adhesive glands.

I wish here to thank Dr. Harold Humm and Dr. Chauncey Goodchild for the constant interest they have shown in my specialty and for their generous expenditure of time and trouble in furnishing me with turbellarian specimens.

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STUDIES ON ARTHROPOD CUTICLE. VIII. THE ANTENNAL CUTICLE OF HONEYBEES, WITH PARTICULAR REFERENCE TO THE SENSE PLATES[1, 2]

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The present study was initiated to investigate the question of whether or not the cuticle over chemoreceptors differs other than in thickness from adjacent areas of the cuticle. Due to the mixed distribution of various types of sensilla there is no case where identification of a particular type of sensillum as a chemoreceptor has been absolutely proven. Of the several cases where strong experimental evidence supports the assigned function of chemoreception (Dethier and Chadwick, 1948), the sense plates of honeybees were chosen for study largely because preliminary investigation showed they were more readily prepared for electron microscope examination than other sensilla. Examinations made about ten years ago showed that probably little useful information would be obtained by electron microscope examination of untreated cuticles overlying chemoreceptors; what was needed was an accessible type that could be readily prepared in order to develop extraction and staining analyses for the electron microscope to parallel studies made with the light microscope. Since a single tangential section of a bee's antenna may show several dozen surface views of sense plates satisfactory for electron micrography, these are favorable if not indeed ideal material for such a study.

The answer obtained to the initial question stated above is that the cuticle over these sense plates is different from the cuticle over other parts of the antenna in more ways than just in thickness. Also, the cuticle differs on different types of sensilla. Little information is available on the nature and significance of these qualitative differences but the relatively low lipid content of the epicuticle and the incomplete sclerotization imply relatively ready penetration. Incidentally, an unexpected and novel sequence of sclerotization changes was encountered.

Methods

For this study, honeybees were mostly collected from hives maintained here. The majority of the work was with the worker caste but, at least for electron micro-

[1] Paper No. 2831, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul 1, Minnesota.

[2] The work reported in the present paper was done under a contract between the Office of the Surgeon General, U. S. Army, and the University of Minnesota. Under the terms of this contract the Army neither approves nor restricts the publication of data.

The author would like to express his indebtedness to Frances H. Korda, Helen T. Y. Lee, C. L. Pu and Marion B. Clausen for considerable amounts of technical assistance at various stages of the work. Chemists of the du Pont Company suggested that $TiCl_4$ and $ZrCl_4$ would react with exposed amine groups on chitosan, and supplied us with samples. Drs. M. H. Haydak and T. A. Gochnauer kindly kept us supplied with bees of desired ages. Thanks are also due to Drs. V. G. Dethier, M. H. Haydak and D. E. Minnich for reading the manuscript.

graphs of sense plates, the fully hardened adult drone is similar. Pupal development requires about nine days, and the adult bee emerges from the pupa within a sealed cell and does not chew its way out of this cell until about a day after emergence. For purposes of the present paper, a "teneral adult" is defined as an adult which has been removed from its pupal cell prior to chewing its way out, and an "older adult" is defined as an adult active outside the hive.

Pupae were seriated in terms of developmental stage without any record being kept of the actual hours of development. Ample material being available, sealed pupal cells were opened at random and preliminary seriation made on basis of the eye color, younger stages being more finely seriated by examining the head capsule under a dissecting microscope. Excessive numbers were taken in the desired ranges and finally seriated after sectioning.

Examination of preparations was made with a Leitz microscope equipped with apochromatic lenses, a Leitz research polarized light microscope, or an RCA electron microscope, model EMU. Some preparations were also examined by phase-contrast microscopy. A considerable variety of techniques was employed. Sections were made free hand from fresh material or with a microtome after embedding either *in vacuo* following freeze-drying or *via* benzene following fixation (usually in Carnoy's or Bensley's fluid but sometimes in Bouin's fluid). For the ordinary light microscope, sections were examined unstained, or after extraction with hot water (100° C.) or 10% NaOH solution, or after staining with Mallory's triple stain, Heidenhain's iron haematoxylin, the argentaffin reaction, the Schiff polysaccharide test, or 2% osmic acid. For the polarized light studies, sections and whole mounts were examined in water both as normals and after treatment with 10% NaOH at 100° C. to purify the chitin, or 60% KOH at 160° C. to convert the chitin to chitosan.

For the electron microscope, free-hand tangential sections were prepared, the cells lysed by brief soaking in distilled water, the inner surface then brushed to wipe off the disintegrating cells, the sections rinsed in distilled water, and then, with or without further treatment, placed on bare electron microscope grids and air-dried. This method of preparation limits the EM examination to a single view—that normal to the plane of the surface. Treatments included extraction procedures similar to those used previously in cuticle studies (Richards and Korda, 1948) and an extensive series of reagents treated in the next section. It was noted that in general, less disruption of extremely thin membranes and ultrafine structures followed purification to chitin when Diaphanol [3] was used than when hot alkaline solutions were employed.

Electron Microscope Staining of Cuticle

Objects are resolved in an electron microscope because of differences in density between the object and its surrounding medium. A common limitation in biological work is that inadequate density differences between adjacent parts result in inadequate contrast in the pictures obtained. In general, this limitation may be overcome by increasing the purification of the material, by shadow-casting, or by inducing the deposition of heavy elements in the material. Purification is of limited value when one is studying the normal structure of a heterogeneous system, and shadow-casting is limited to clarifying surface irregularities (best with one type

[3] A saturated solution of chlorine dioxide in 50% acetic acid.

TABLE I

Electron microscope staining reactions with various cuticle samples

Aqueous sols.	% conc.	Chitin *Periplaneta* trachea	Peritrophic membrane *Phormia*	Larval cuticle *Aedes*	Trachea *Periplaneta* adult	Trachea *Phormia* adult	Trachea *Calandra* adult	Crop lining *Phormia*	Crop lining *Periplaneta*	Wing membrane *Aedes*	Wing scales *Aedes*	Tracheal airsac honeybee	Sense plates honeybee
$FeCl_3$	2	—	sl. Gr.	—	?	?	—	?	?	Gr.	—	sl. Gr.	—
$ZrO(NO_3)_2$	0.1–5	—	v. Gr.	v. Gr.	Gr.	sl. D.	—	sl. Gr.	sl. D.	sl. Gr.	—	sl. Gr.	sl. Destr.
$AgNO_3+NH_4OH$	*ca.* 5	—	?	—	var. Sp.	v. l. Sp.	—	—	?	v. sp.	v. l. Sp.	—	f. Sp.
$H_3PW_{12}O_{40}$	2+6	Destr.	?	?	v. Retic.	sl. D.	?	Gr.	?	—	—	var. Gr.	Gr.
OsO_4	2	—	—	sl. D.	v. D.	sl. D.	?	?	sl. D.	v. D.	v. D.	v. D.	sl. D.
$IrCl_4$	1	—	v. Gr.	v. Gr.	sl. Destr.	sl. Gr.	sl. Gr.	sl. D.	sl. Destr.	?	?	v. Gr.	—?
K_2PtCl_6	2	—	sl. Destr.	—	—	—	—	?	?	—	—	sl. Gr.	—
$HgCl_2$	Sat.	—	?	Var.?	—	—	—	—	?	—	—	sl. Gr.	—
Millon reagent	—	—	—	—	—	—	—	—	sl. Destr.	—	—	—	?
$Pb(C_2H_3O_2)_2$	2	—	sl. D.	—	D.	—	?	—	?	—	—	—	—
$Th(NO_3)_4$	2	—	sl. D.	Gr.	v. Retic.	sl. D.	sl. D.	sl. D.	sl. D.	sl. Gr.	—	Gr.	?
$UO_2(NO_3)_2$	2	—	?	?	—	—	—	sl. D.	?	—	—	var. Gr.	—
$NaZn(UO_2)_3$ $(C_2H_3O_2)_9$	2	—	sl. D.	sl. Gr.	v. Retic.	?	sl. Gr.	sl. Gr.	?	sl. Gr.	?	Gr.	—

— = negative; ? = negative or questionable slight effect; D. = distinct darkening, more or less general; Gr. = granular appearance to membrane; Sp. = distinct separated spots; Retic. = spots connected by streaks giving reticulation; Destr. = some structural destructive changes.

v. = very; sl. = slight; f. = fine or minute; l. = large; var. = variable or inconsistent.

of surface discontinuity on an otherwise plane surface; shadow-cast specimens were examined but did not facilitate study of these sense plates). Accordingly, a considerable amount of effort was expended in an attempt to develop a useful set of "stains" which would augment contrast *within* a specimen of cuticle and perhaps even be sufficiently specific in action to aid in cytochemical identifications (Richards, 1952a). The results were discouraging but not nil.

Some of the more interesting data are presented in Table I. These results were obtained after cleaning cuticles in distilled water and then soaking in the reagents at the indicated concentration (usually two days), excess of reagent being removed by washing in water prior to drying on an electron microscope grid. All tests were both replicated and repeated. Inconstancy in thickness of preparations, even in only slightly separated parts of the same membrane, makes it impossible to distinguish between truly negative effects and a possible uniform deposit by comparing control and treated membranes. But structural variability is much more serious than variations in thickness; recordable differences are limited to relatively gross qualitative differences (see Richards and Korda, 1948).

Since no two of the membranes selected as test objects gave identical results, the data emphasize that a considerable amount of interspecific variation is to be expected in the details of cuticle chemistry. The antennal sense plates are not identical in reactions to any of the other membranes tested, but they appear in these tests to be more similar to the cuticle samples from *Phormia* than to the tracheal airsac walls or, as will be brought out in later sections, other cuticularized areas of the same species.

The osmic acid effect (Fig. 23) is negative after extraction of the membrane with hot chloroform. At least for the honeybee airsac it is also negative after exposure to intense electron bombardment (see Hillier *et al.*, 1950). Occasional breaks in the specimens show that the effect is localized in the epicuticle but no separable osmium-positive layer was recognized. Broken preparations of bee airsacs show two principal and separable layers; both the osmic acid and the iridium tetrachloride effects are limited to the outer of these layers but the outer layer itself is still present after refluxing with chloroform and will still react with the iridium chloride. In fact, series of tests with cockroach tracheae, bee airsacs, bee sense plates and mosquito wings and scales showed that extraction with hot chloroform interfered only with the osmic acid reaction. Presumably the osmium is reduced by only the lipids in the cuticles used for these EM tests, and the other compounds react with non-lipid components.

The ammoniacal silver nitrate effect is the standard argentaffin reaction positive for polyamines, polyphenols and certain aldehydes. In cuticle work, a positive reaction is presumed to be due to the presence of a polyphenol substrate for sclerotization (Richards, 1951). The degree of localization of the polyphenol by means of this test is not known but the spots of silver (Fig. 20) are clearly discrete in contrast to the diffuse deposition of osmium (Fig. 23).

Except for the osmic acid and ammoniacal silver nitrate reactions, no explanation can be given for the staining of cuticle by compounds listed in Table I and illustrated in Figures 20–23. Obviously, however, the reactions are not simple ion effects, for the nitrate of thorium is excellent but the chloride is not, and of the three uranyl compounds tested, only the complex one gave good pictures. Several routine histological reagents (chloroplatinic acid, mercuric chloride) were either

negative or so light and general that the effect was not visible. Aqueous solutions of all of the compounds in Table I are acidic except for the ammoniacal silver nitrate which is close to neutrality. Phosphotungstic acid is a general protein precipitant that deposits in at least most of the cuticles used but the alkaline solutions of sodium and potassium tungstate are ineffective; however, many of the ineffective compounds form acidic aqueous solutions and may even be known protein precipitants.

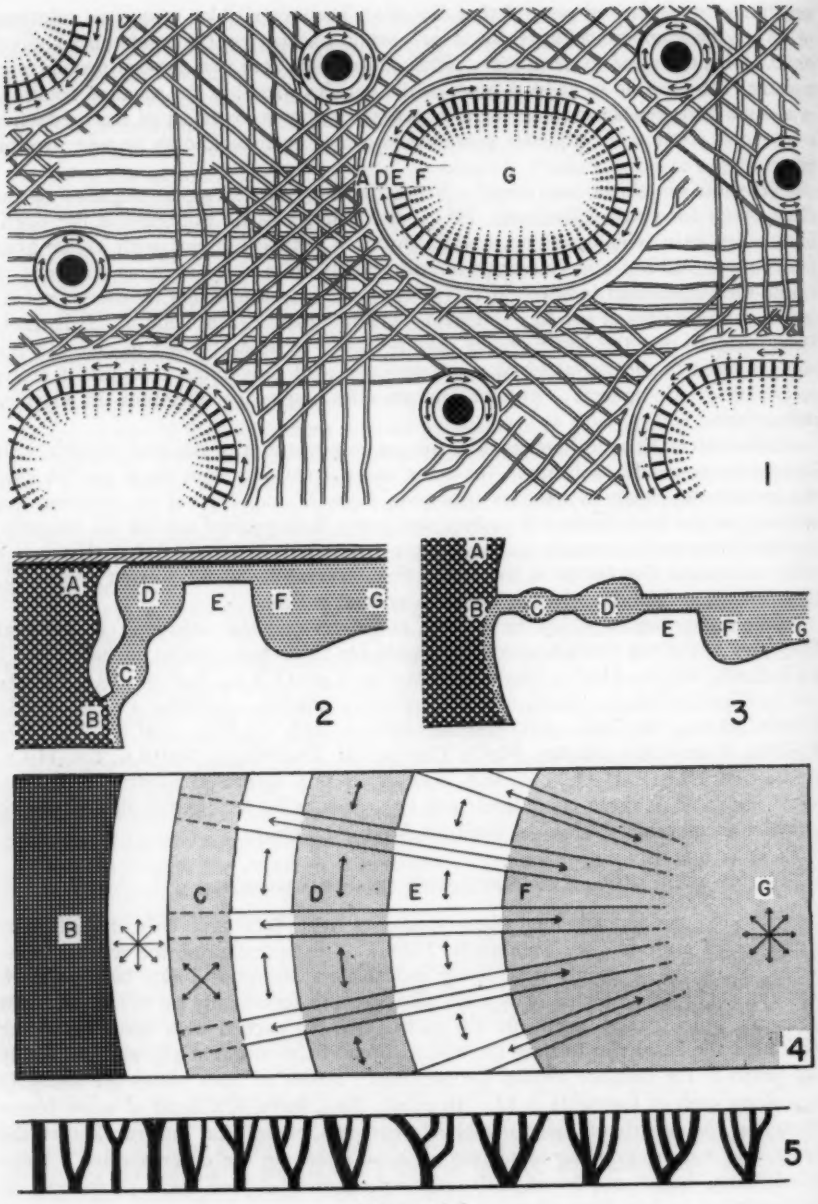
No stain for chitin was found. Since the honeybee airsac is negative to the classic tests for chitin (Richards, 1951), the EM stains do not suggest the possibility of a stain which will react with chitin in cuticle but not with chitin after purification. Chitin and chitosan are both visibly colored by aqueous solutions of iodine plus potassium iodide, but in the electron microscope this treatment results only in a slight overall increase in density which does not seem helpful. It is interesting to note that the chitin chains may not only be de-acetylated to chitosan but also oxidized to the polyaldehyde with periodic acid (involving removal of entire acetylamine side groups) without the micelles appearing different in electron micrographs.

Chitosan can be readily stained by the use of zirconium or titanium tetrachloride in anhydrous organic solvents.[2] In some cases, treatment with these can be used to augment the clarity of micelles (compare Figs. 27 and 29) but the pretreatment required to produce chitosan is violent and leaves little general use for the reagents. Applied to normal cockroach tracheae, these compounds had a somewhat destructive effect or caused dissolution of the endocuticle, depending on the solvent employed; they seemed to give no staining effect to normal cuticle.

Other compounds which have been tested as aqueous solutions (usually at 2%) and found not promising as EM stains for these test cuticles include: NaCl, KCl, $CaCl_2$, $CrCl_2$, $MnCl_2$, $MnSO_4$, $KMnO_4$, $K_3Fe(CN)_6$, $K_4Fe(CN)_6$, $FeSO_4$, $CoCl_2$, $Na_3Co(NO_2)_6$, $NiCl_2$, $CuSO_4$, $(NH_4)_6Mo_7O_{24}$, $AgNO_3$, $CdCl_2$, $SnCl_4$, $KSbOC_4H_4O_6$, K_7TeO_3, KI, H_5IO_6, KIO_4, CsCl, Cs_2SO_4, $BaCl_2$, Na_2WO_4, K_2WO_4, $KAu(CN)_2$, $HgBr_2$, HgCl, $TlNO_3$, TlI, $Pb(NO_3)_2$, $NaBiO_3$, $BiC_6H_5O_7$, $ThCl_4$, and $UO_2(C_2H_3O_2)_2$. $SbCl_3$ was tested as a saturated solution in chloroform. Several of these compounds are to a greater or less extent destructive to cuticular membranes ($KMnO_4$, $SbCl_3$, $KAu(CN)_6$, etc.).

General Description of the Antennae

The antennae and antennal sense organs of honeybees have been described by a number of authors (see Snodgrass, 1925). The antennae of the worker caste consist of twelve segments with freely movable membranous joints between each segment. The sense plates or sensilla placodea are found only on the distal eight segments, but on these segments are rather densely packed in a band extending from near the basal rim to near the apical rim and circumferentially approximately two-thirds of the distance around the segment. Bands of sense plates are similarly located on each of segments 5–12. In effect, then, there is a band of sense plates extending the length of these combined segments except for interruption at the joints, and a corresponding band free of sense plates on the outer surface. Setae called "sense hairs" are found both scattered around between sense plates and on the band that is free from sense plates. Other types of sensilla are more local in



FIGURES 1–5

distribution. It has been estimated that the antenna of the worker caste has 5000–6000, those of drones about 30,000 sense plates, and that the total number of antennal sense cells is of the order of 500,000 (Snodgrass, 1925).

We can use the above facts, plus data to be presented, as basis for recognizing six types (or areal types) of cuticle on the antenna: (1) sclerotized cuticle between sense plates, (2) sclerotized cuticle in the band devoid of sense plates, (3) sclerotized cuticle devoid of sense plates and setae and forming the basal and apical rims of each segment, (4) soft or unsclerotized cuticle on the intersegmental joints, (5) sclerotized cuticle of sense hairs and sense pegs, and (6) less sclerotized cuticle on sense plates and "sunken setae." Any of these may be compared with cuticle from other parts of the body—such comparison showing that the unorthodox sclerotization of the antennae is not found in the general cuticle of thorax and abdomen and hence is peculiar to the antennae as such, rather than to the honeybee as a species.

Actual presentation of the data, however, will be simpler or at least less redundant if we treat (1) the chitin chain or micelle orientations in the general antennal cuticle, (2) orientations in setae, (3) general structure and orientations in sense plates, (4) development of the epicuticle, (5) development of the procuticle, (6) comparison of the cuticle on various types of sensilla, and (7) comparison to thoracic and abdominal cuticle.

Micelle Orientations in the General Cuticle

Like arthropod cuticle in general (Richards, 1951), the antennal cuticle is laminated. But the laminae in the bee antennae are thin (ca. 0.8 μ) and so low in contrast that they cannot be readily seen in routine observation except at the antennal joints where the cuticle shows a light staining with fuchsin between the thickened laminae (Fig. 31). Unstained sections examined by dark phase-contrast microscopy reveal a general laminate structure. Laminations have been discerned in some sections of frozen-dried preparations stained with haematoxylin. Laminations may also be inferred from alternating lighter and darker bands of birefringence in sections treated with hot water or alkali (Fig. 8), from the presence of crossed Balken layers (see below), and from rows of spots along pore canals of antennae treated with ammoniacal silver nitrate (Fig. 12). The fully developed cuticle is approximately 15 μ thick; accordingly there are some 15–20 laminae.

In the normal, fully developed cuticle no macrofibers or Balken can be seen

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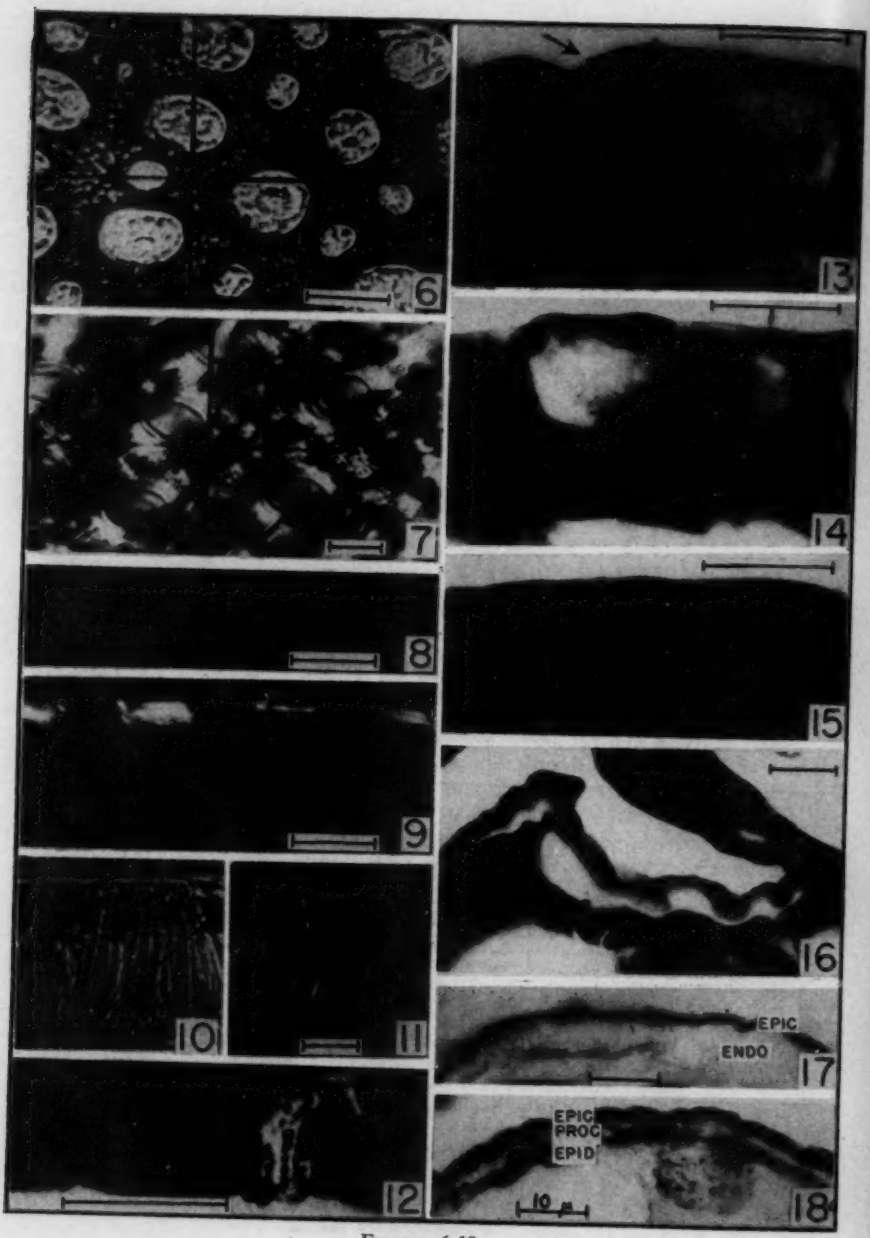
Figure 1. Semi-diagrammatic sketch of a small area of an antennal segment showing distribution of sense plates and sense hairs (sockets as cross-hatched circles), orientations of Balken as seen in specimens purified for chitin, and orientations of micelles in sockets as indicated by arrows. Lettering indicates corresponding parts in Figures 1–4.

Figure 2. Diagrammatic section of normal relations of the cuticle of half a sense plate, its rim or socket, and the adjacent cuticle.

Figure 3. Similar diagrammatic section showing opening out of structure, concomitant with drying, after rupture or removal of the epicuticle. Needed for interpretation of some of the electron microscope pictures (Figs. 19 and 25).

Figure 4. Map of micelle or chitin chain orientations in the cuticle of a sense plate and its socket.

Figure 5. Types of ribs or Balken seen in electron micrographs of the thin membrane between sense plates and the socket rim (see Figs. 19 and 22).



FIGURES 6–18

clearly (Fig. 6), and the cuticle seems nearly isotropic (Fig. 9) (except for setae and sockets, see next section). If the cuticle is treated with water at 100° C. for several days or, better, the chitin purified or further changed to chitosan, a rather coarsely fibrous structure is revealed. These fibers, commonly called Balken, average about 0.5 μ in diameter (range 0.3–1.0 μ), have a more variable spacing which, however, roughly approximates the fiber diameter, and are strongly birefringent with sharp extinctions (Fig. 7). As shown in Figure 1, where for clarity of the diagram the fiber diameters are reduced and the spacings between fibers exaggerated, these fibers occur in layers (Balkenlagen) which apparently correspond to laminae of the cuticle. Four orientations can be recognized forming a crossed-fiber type of orientation: one is longitudinal, one transverse, and two oblique at roughly a 40° angle with the longitudinal set. It was not found possible to count the number of superimposed Balken but since sections are similarly birefringent from outer to inner surface (Fig. 8) the number may well be the same as the total number of laminae. Although four orientations could be readily recognized, the sequence of these was not determined because of technical diffi-

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Magnification of individual photographs indicated by a 10 μ line on each picture.

FIGURE 6. Free-hand tangential section of an antenna fixed in Bensley's fluid and examined unstained in water. Shows distribution of sense plates (large ovals), sense hairs (smaller circles) and pore canals (very small spots).

FIGURE 7. Free-hand tangential section of an antennal cuticle purified to chitosan with hot conc. KOH. Photographed between crossed Nicol prisms with a rotating compensator set to accentuate birefringence along one diagonal while reducing it along the other. Two crossed sets of diagonal Balken are shown. Compare with Figures 1 and 6.

FIGURE 8. Longitudinal section of an antennal cuticle purified for chitin in a 10% NaOH solution at 100° C. Photographed between crossed Nicol prisms.

FIGURE 9. Longitudinal section of an antenna fixed in Bensley's fluid and examined unstained in water. Photographed between crossed Nicol prisms.

FIGURE 10. Long and short sunken setae in a longitudinal section of an antenna fixed in Bensley's fluid and examined unstained in water.

FIGURE 11. Same photographed between crossed Nicol prisms showing the strong, sharply extinguishable, birefringence of socket walls and shaft.

FIGURE 12. Longitudinal section of an antenna that had been abraded with emery powder and then soaked in ammoniacal silver nitrate solution. The thinner parts show precipitation of silver in the pore canals between cuticular laminae.

FIGURE 13. Longitudinal section of an antenna which had been frozen at − 195° C., dried by sublimation at − 35° C., and then embedded in paraffin *in vacuo*. Stained with Mallory's triple stain. Arrow shows area where the epicuticle is separated from the surface of a sense plate.

FIGURE 14. Longitudinal section of an antenna fixed in cold Bouin's fluid and stained with Heidenhain's haematoxylin. Shows differentiation of the sense plate socket and of the epicuticle.

FIGURE 15. Another photograph from the same section but from the region where sense hairs but no sense plates are found.

FIGURE 16. Section of a pupal antenna stained with Mallory's triple stain and photographed with a green filter. Shows the future membrane between antennal segments, and developing sclerotization at each end of this membrane.

FIGURE 17. Section of the membrane between antennal segments of a teneral adult stained with the periodic acid-Schiff reagent method for polysaccharides. Epicuticle intense red; endocuticle faint pink.

FIGURE 18. Similar to preceding but from a late pupa. Epicuticle intense red; procuticle and epidermal cells light pink.

culties arising from the fact that after alkali treatment one set of fibers droops into the interstices of the next. It can be recorded that in all of the several dozen specimens examined, the outermost layers of fibers were oblique.

At least the outermost sets of Balken fuse into a rim around the sockets of setae and other sensilla. Presumably lower layers do likewise. Here and there a Balken fiber can clearly be seen to branch but without any constant angle being involved; some gradually separate at a small angle, others branch off almost at a right angle and then turn abruptly to become parallel.

There seem to be two general possibilities for the origin of Balken fibers: either they are giant micelles or they are micellar aggregates (Richards, 1951). Branched Balken of diverse diameters, illustrated by Langner (1937) for diplopod cuticles, favor the idea of micellar aggregates (also, Biedermann (1903) reported that Balken seem to be composed of more minute fibers). This is strongly supported for the Balken of honeybee antennae by the fact that electron micrographs of the frayed edges of antennal sections (Fig. 28) sometimes show Balken separated into smaller fibrils that extend down to micellar dimensions (Richards and Korda, 1948). Further support for the idea that Balken are micellar aggregates comes from the purely chemical separation of "submicroscopic Balken" of sense plates into fibrils of micellar dimensions (Figs. 27, 29). These data are consistent with Kühnelt's (1928) suggestion that Balkenlagen are only specialized cuticular laminae. No evidence was obtained as to why successive laminae show different orientations and so produce the crossed-fiber type of structure.

The Balken sets extend around the antennal segment and appear entirely similar in the bands with and without sense plates. However, at the ends of each antennal segment the Balken lose their identity in the basal and apical rings. These areas appear to lack a Balken type structure, or, if one prefers, to be a single larger Balken (since they are an indivisible band of tightly packed, paralleled molecular chains). These apical rings show sharp extinction with the chitin micelles extending circumferentially (= tangentially) around the antennal segment.

The author was somewhat surprised to find the optical effects showing that the soft intersegmental membranes of the antennae have closely paralleled micelles. In this case there seems to be no tendency for the micelles to aggregate into Balken —at least the membrane appears uniformly birefringent. The sharp extinction shows that this membrane does not have a crossed-fiber type of structure; the micelles are parallel to the axis of the antenna and hence at a 90° angle to those they join at the apical and basal ends of each segment.

Longitudinal sections show a laminate structure between crossed Nicols due to different amplitudes of birefringence in successive laminae (Fig. 8). A similar picture from tick cuticle has been published by Schulze (1932). Such an effect is to be expected from any single plane through sets of crossed fibers even though each lamina would be equally birefringent if properly oriented.

The size and spacing of the relatively large pore canals (Fig. 6) is consistent with their passing between Balken and through the interstices of crossed Balken sets.

Micelle Orientations in Setae

The cuticle of setae ("sense hairs") and setal sockets, unlike the general surface cuticle, is distinctly birefringent in its normal state (Figs. 9, 11). The explanation

of this difference is not known although several suggestions are possible (see Richards, 1951, p. 84). The amplitude of setal birefringence can be increased by purification of the chitin.

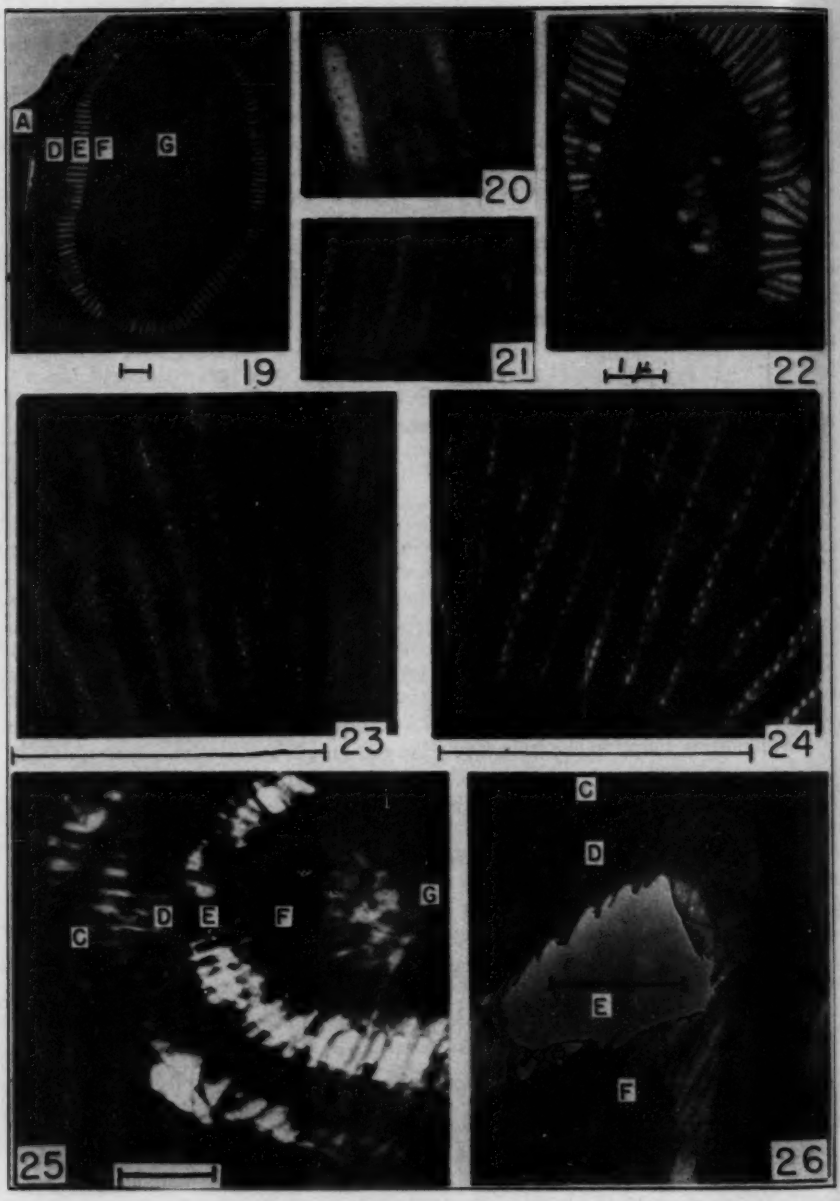
Both the sense hairs and their socket rims show sharp extinction indicating a high degree of orientation. The micelles are parallel to the setal axis along the shaft, radial in the thin soft socket membrane, and circumferential or tangential in the socket rim. The shaft of sense pegs is similar but the sockets show less birefringence (details of which were not determined). Both long and short sunken setae (Figs. 10–11) show orientations similar to those of tactile setae; since the tormogen membrane is, in effect, stretched and elongated to form the setal pocket, the radial micelles of this membrane are also oriented approximately parallel to those of the setal shaft.

Clearly, micelle orientations do not depend on sclerotization for stability because equally sharp extinctions are given by soft membranes (staining blue with Mallory's stain), by incompletely sclerotized membranes (red with Mallory's stain), and by heavily sclerotized membranes (refractory to stains). The extensive analysis of *Drosophila* setae by Lees and Picken (1945) suggests that growth pressures produce the longitudinal orientation in setal walls while they are being laid down.

The General Structure and Micelle Orientations of Sense Plates

The sense plates, or cuticles thereof, are thin complicated membranes overlying a group of cells that project most of the way through the cuticle (Figs. 33, 46). In surface view (Figs. 1, 6, 19) they are seen to be more or less oval structures whose long axes tend to parallel the axis of the antenna. A central plate (Fig. 1, *F–G*) is surrounded by a very thin membrane (*E*) which joins a thick rim (*D*) which in turn is surrounded by a socket rim of the general cuticle (*A*) formed by the fusion of Balken. The sensillum attains this recognizable differentiation very early in cuticle development (Figs. 38–39) but the following notes are based entirely on the condition found in the fully formed organ on adult antennae. Some authors have suggested that these peculiar structures can be derived, in a phylogenetic sense, from setae by assuming that the setal shaft is flattened down to become the central plate (Snodgrass, 1925).

In cross or longitudinal section (Fig. 2), it is seen that a double-layered epicuticle extends continuously across the sense plate (*D–G*) and adjacent cuticle (*A*). Beneath the epicuticle is a continuous chitin-protein cuticle. This is only partially sclerotized in the sense plate and its rim (*C–G*) but heavily sclerotized in the surrounding cuticle (*A–B*). If the epicuticle is removed or disrupted and the sense plate caused to shrink under electron bombardment, the structure straightens out in the manner diagrammed in Figure 3. There is no question that the rim is firmly attached to the cuticle at *B–C* (this can occasionally be seen in stained sections examined with the light microscope too), but it is not certain whether the chitin micelles fuse into those of the rim at this point or extend inwardly to form a lining of the cavity which contains the cells of the sensillum. It is also uncertain whether the space diagrammed between *A* and *C–D* is a real space or only a line of weak linkages; it does, however, coincide with a line of differentiation demonstrable with either Mallory's or Heidenhain's stains (Fig. 14).



FIGURES 19–26

Examination with an electron microscope reveals that the thin membrane is traversed by some 120–150 radially arranged thickenings (Fig. 19). Over 80% of these are simple rods, well over 90% are either simple or bifurcated rods, but a small percentage of triply, quadruply or irregularly branched ones are seen (Fig. 5). Examination of extracted specimens shows that these thickenings are rod-like structures that may be compared to the Balken of the general antennal cuticle from which they appear to differ only in being beyond the limits of resolution by visible light microscopy. These "submicroscopic Balken" extend centrally into the middle of the sense plate (Fig. 1) and then lose their radial orientation (Fig. 22); they also extend through the rim *D* and on at least to *C* maintaining their radial orientation (Figs. 25–26). By prolonged and rather violent chemical treatments these Balken can be broken into microfibers of micellar dimensions (Fig. 29) and "stained" with zirconium or titanium to increase their contrast (Fig. 27).

In addition to the radially arranged micelles aggregated into Balken, there are circumferential (= tangential) micelles at least in areas *D* and *E*. For the rim *D* this is shown by polarized light effects. Figure 7 shows that the rims are birefringent in surface view; Figure 9 shows that the rims are also birefringent along radii of the sense plates but isotropic where the rim is cut in cross section. Accordingly, since the optic axis of chitin micelles is known to parallel the fiber axis (Richards, 1951), the micelles must be circumferential. Electron micrographs not included among the illustrations for the present paper indicate that a single layer of circumferential micelles extends around the membrane *E* and across the radially arranged Balken (specimens treated with iridium tetrachloride). It seems highly probable that the rows of holes seen in the membrane between Balken in Figures 21, 23 and

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Magnification of individual electron micrographs indicated by a 1 μ line on most of the pictures.

FIGURE 19. Relatively low power micrograph showing structure of the cuticle of a sense plate from a preparation cleaned in distilled water at room temperature (= normal). Lettering as in Figures 1–4.

FIGURE 20. Micrograph of a small portion of the thin membrane area (region *E* of Figs. 1–4 and 19) after soaking in ammoniacal silver nitrate solution (2 days). The dark spots represent silver deposits of the order of 100–200 Å. Magnification approximately equal to that of Figure 24.

FIGURE 21. A similar area of thin membrane after soaking in a 5% solution of phosphotungstic acid or $H_3PW_{12}O_{40}$ (1 day). The light spots appear to be breaks in the epicuticle between circumferential micelles (see text). Magnification as for preceding.

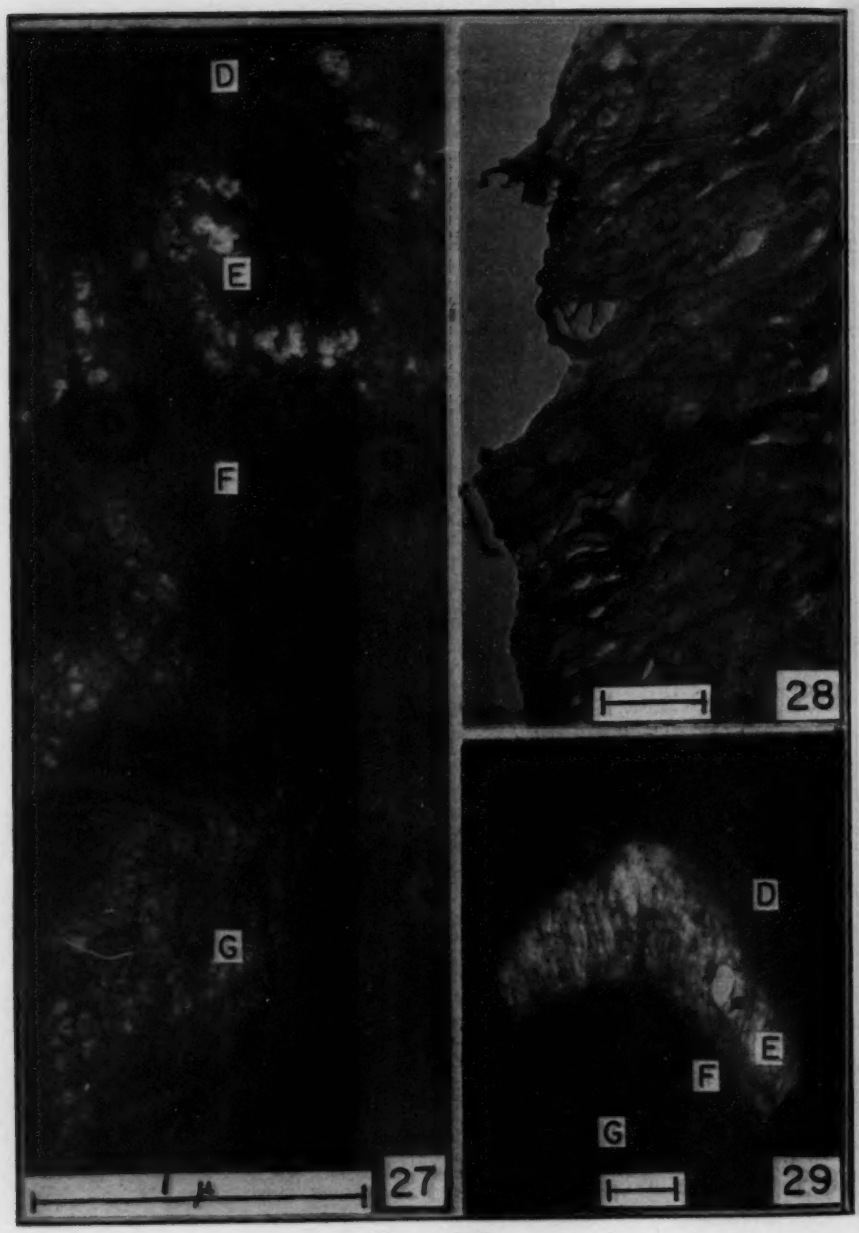
FIGURE 22. Another relatively low power micrograph of the cuticle of a sense plate after extraction in distilled water at 100° C. (1 day). Shows dispersion of a central area of the plate revealing thickenings.

FIGURE 23. A small portion of the thin membrane after soaking in a 2% solution of osmic acid or OsO_4 (1 day). The light spots are similar to those in Figure 21 (see text).

FIGURE 24. A small portion of the thin membrane after soaking in Diaphanol (3 days) following initial cleaning in distilled water and refluxing with chloroform.

FIGURE 25. One end of a sense plate after soaking in 30% H_2O_2 (7 days). The field of view represents the areas from *C* to *G* of Figures 2–4. Note the continuity of the thickenings (Balken) from *C* through *F*.

FIGURE 26. One end of a sense plate after soaking in Diaphanol (8 weeks). Shrinkage and electron destruction is obvious but note the cross striations on and between the radial thickenings.



FIGURES 27–29

24 represent spaces between such circumferential micelles,[4] and that the cross striations between radial thickenings in Figure 26 do likewise.

A composite survey of the data on micelle orientations is given in Figure 4. Recapitulating, evidence for tangential orientation in the thick rim *D* is based on optical effects; radial orientation in *E–F* is based on both optical effects and electron micrographs; all other orientations indicated are based entirely on electron microscope pictures.

Dimensions of the various portions of sense plates are: the overall surface size averages 10 × 14 μ; the central plate averages 7.5 × 11 μ, and is 0.5–1.25 μ thick at the center (*G*) and 1–2 μ thick at the edge (*F*); the thin circumferential membrane normally appears to be about 0.6 μ broad in surface view but displaced sensilla and many electron microscope pictures show it as 1–1.5 μ broad (apparently due to the membrane actually extending part way across the rim *D*). The Balken in this membrane average 0.1 μ in diameter when dry with a range of 0.08–0.15 μ except for a few branched ones where the common trunk is as large as 0.2 μ. The membrane between these Balken (excluding the epicuticle which is several tenths of a micron thick) is only a few hundred Ångstrom units thick when dry; how thick it may be in the living insect is unknown but it can scarcely be more than a few tenths of a micron.

The Epicuticle and Its Development

The epicuticle forms a continuous, slightly birefringent outer sheath over the antenna, but being only a fraction of a micron thick, it is difficult to study. Its continuity without interruption across the sense plate from the surrounding cuticle (Fig. 2) is shown by certain slides, prepared by the freeze-drying technique, in which the epicuticle became separated from the underlying chitin-protein layer at some places (Fig. 13); continuity is also indicated by certain broken preparations which, when examined in the electron microscope, show a thin layer that seems certainly epicuticle projecting without apparent discontinuity from the sense plate, the surrounding cuticle and the minute intervening space. Apparent continuity can also be observed in ordinary stained sections viewed with a light microscope (Fig.

[4] In Figures 21 and 23 the epicuticle has apparently broken under electron bombardment where most strained and least supported, *i.e.*, between the circumferential micelles; in Figure 24 the epicuticle has been removed chemically. These spaces are illustrative of one of the most serious technical difficulties involved in electron microscopy of insect cuticle, namely, that cuticle (and chitin) shrinks a considerable amount under electron bombardment (and may be readily "burned up" or distorted beyond recognition if the electron beam is made intense).

Magnification of individual electron micrographs indicated by a 1 μ line on each picture.

Figure 27. Portion of a sense plate after treatment, successively, in dist. H_2O, 30% H_2O_2 (9 days), 60% KOH at 160° C. (20 min.), and alcoholic 5% $TiCl_4$ (30 min.). Titanium reduced onto exposed amine groups acts as an electron stain to increase the clarity of the micelles. Lettering as in Figures 1–4 and 25–26.

Figure 28. Frayed end of a section of sclerotized cuticle between sense plates after treatment with 10% NaOH at 100° C. (1 day). The bracket indicates a single Balken visible with the light microscope.

Figure 29. One end of a sense plate after soaking in 60% KOH at room temperature (3 days) and then heating to 160° C. (15 min.). Shows separation of the radial thickenings into smaller fibrils of somewhat variable width, and, in comparison with Figure 27, low contrast correlated with the absence of an electron stain.

14) but, as Blower (1951) has emphasized, diffraction effects along the cuticle edge may create serious illusions in work on the epicuticle.

The epicuticle was not actually separated into two layers by any of the methods employed but it appears nonetheless double, at least in the sense that the outer layer or outer surface is stainable with osmic acid and Heidenhain's haematoxylin (Figs. 14–15). In comparison to the epicuticle over the general surface of the antenna, the epicuticle seems to be thicker on the intersegmental membranes (Figs. 16–18) and thinner across the sense plates but the thicknesses are too small for accurate measurement in these sections.

Data on the stainability of the epicuticle of various antennal areas at several developmental stages are summarized in Table II. In very early stages of devel-

TABLE II

Changes in staining reactions of the epicuticle during development as seen with the light microscope. Upper term refers to outer layer (when distinguishable), lower term to inner layer of epicuticle; dash indicates refractory to staining by the indicated method

	Violet eyes, colorless antennae =Figure 36			Purple eyes, yellowish antennae =Figure 39			Teneral adult			Older adult		
	I.M.	S.P.	Scl.	I.M.	S.P.	Scl.	I.M.	S.P.	Scl.	I.M.	S.P.	Scl.
Schiff polysaccharide test	Red	Red	Red	Red Pink	Red Pink	Red Pink	Red Pink	Pink —	— —	Red Pink	— —	— —
Argentaffin test				Spots? —	Spots? —	Spots? —	— —	— —	Gray —	— —	— —	Gray —
Osmic acid 2%				— —	— —	— —	— —	— —	Black —	— —	— —	Black —
Mallory's triple stain	Red	Blue	Blue	Red Red	Red ?	Red —	Red Pink	Purple —	— —	Red Pink	Purplish —	— —
Heidenhain's iron haematoxylin				Brown Black	Brown Black	Brown Black	Gray —	Black —	Gray —	— —	Gray —	Black —

I.M. = intersegmental membrane between antennal segments.
S.P. = sense plates on antennae.
Scl. = sclerotized general surface of antennae.

opment in the pupa it is extremely difficult to differentiate between the epicuticle and a possible precipitate of the molting fluid on the surface (Figs. 35–38). Mallory's stain colors both epicuticle and molting fluid blue on the future sclerotized areas and gives differentiation only at the future intersegmental region where the epicuticle is colored red, but the Schiff polysaccharide test (Fig. 18) fails to stain the precipitated molting fluid. During these early stages there appears to be a definite membrane (blue with Mallory's stain) outside the developing adult cuticle (Figs. 36–38). The nature of this membrane remains to be studied but the fact that developing sense hairs and sense pegs seem to push into it or push it outward suggest that it is not a part of the forming adult cuticle. Perhaps it is the same as an "ecdysial membrane" found by C. M. Williams and V. Passoneau (unpublished) in *Cecropia* pupae—they consider it to be an undigested innermost lamina of the pupal cuticle.

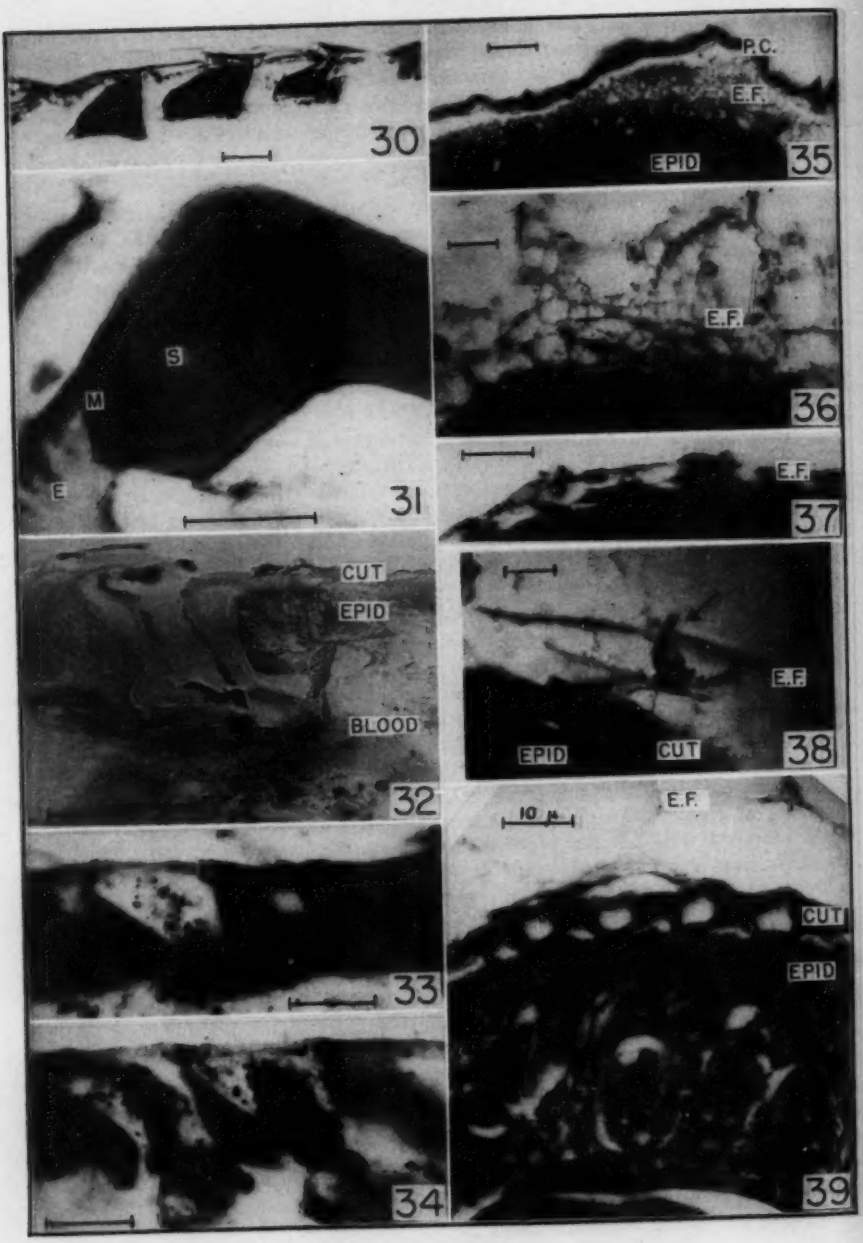
There is even more uncertainty about the interpretation of staining obtained with the argentaffin reaction; clearly the surface precipitate in Figure 32 is considerably thicker than the entire epicuticle, and clearly the silver deposits revealed by electron microscopy are more restricted than the sclerotization effect (Fig. 20 and Richards, 1951, Fig. 64B)—the data make one wonder about the degree of localization obtainable with the argentaffin reaction but we lack detailed knowledge of the chemical reactions involved.

Data presented in Table II show that the epicuticle at first stains uniformly over the antenna. An early differentiation giving different isoelectric points in different areas is implied by the results with Mallory's stain. Subsequently, differentiation results in the ability to recognize at least three types found respectively on the intersegmental membrane, the general sclerite surface, and the sense plates (Richards, 1952b). With Mallory's stain, Heidenhain's haematoxylin and the Schiff polysaccharide test, the change with time is due to partial or complete masking of the reactions given by early stages; with the argentaffin and osmium tests, the change is due to initiation of a positive reaction.

The precipitation of osmium from a 2% solution of osmic acid is detectable with the light microscope only on the general sclerite surface. However, use of the electron microscope reveals a general but very light deposit across the sense plate membranes (Fig. 23). This osmium reaction is prevented when the preparation is previously extracted with hot chloroform. It follows that a very small amount of lipid is to be found in the epicuticle across sense plates. Comparison of osmium-treated preparations with chloroform-extracted ones failed to reveal any recognizable lipid or wax layer, but it should be remembered that the electron microscope studies were limited to surface views where superimposed layers can be distinguished with certainty only when one layer ruptures and peels away from the other.

It has already been mentioned in a previous section that the epicuticle of the thin membrane over sense plates may show rows of minute holes (50–300 Å) after certain treatments (Figs. 21, 23). No such perforations are to be seen in normal preparations with nearly the same resolution. It is thought by the author that these holes in treated epicuticles are produced by the destructive effect of electron bombardment at points of lesser support by underlying circumferential micelles in the chitin-protein layer. Similar holes are to be found in preparations from which the epicuticle has been removed (Fig. 24). There is nothing to indicate that such holes are to be found in normal membranes.

The recognizable subdivisions of the epicuticle have been referred to by the noncommital terms "outer" and "inner." The inner subdivision develops a natural amber color and early becomes resistant to staining; it apparently is the same as the "cuticulin layer" of Wigglesworth (1947, 1948) and the "protein epicuticle" of Dennell (1946). No pore canals (readily stained in the procuticle) could be traced into this layer at any of the stages examined. The outer subdivision is stainable with haematoxylin, osmic acid and ammoniacal silver nitrate; it accordingly is thought to contain protein, lipid and polyphenol but as far as could be determined these occur together, not in separate superimposed layers. The outer subdivision, then, compares with Dennell's "lipid epicuticle" and with Wigglesworth's "polyphenol layer" plus "wax layer." By definition, no tectocuticle (cement layer) can be present since no dermal glands are to be found (also, no layer was separated off the surface by heating in chloroform).



FIGURES 30–39

The Chitin-Protein Cuticle and Its Development

When the adult cuticle begins to develop it is at first a single thin layer that represents the epicuticle (Fig. 36). Within the next few hours there is a rapid increase in thickness, involving the epicuticle becoming visibly double and initiation of production of the chitin-protein procuticle (Richards, 1951). Fortunately for the present study, the sense plates become recognizable very early and can be seen to have already differentiated into central plate, circumferential membrane and rim by the time the cuticle is 1–2 μ thick (Fig. 38). Further development to full thickness (ca. 15 μ) occurs within a day, and events leading to sclerotization commence while the procuticle is still increasing in thickness.

Before treating the unusual details of sclerotization in these antennae, mention should be made of a paper now in press by Schatz (1952) proposing a terminology that will be followed here. Studying sclerotization in various insects, using primarily the differential coloring obtained with Mallory's triple stain (supplemented by haematoxylin and the Millon reaction), Schatz concluded that differentiation of the original soft procuticle is at least descriptively a two-stage process, and that the differentiation results in three rather than two recognizable subdivisions of the chitin-protein portion of the cuticle. The original transparent procuticle stains blue with Mallory's; a portion of this, while remaining transparent, changes to staining red with Mallory's; a portion of this last subsequently becomes refractory to staining and simultaneously develops its own amber or brown color. Classically, the hard colored part is called "exocuticle," the softer, transparent part "endocuticle."

Magnification of individual photographs indicated by a 10 μ line on each picture.

Figure 30. Unstained longitudinal section of the cuticle from a split antenna extracted in dist. H_2O at 100° C. (2.5 days). Shows failure of the treatment to remove the pigmentation.

Figure 31. Longitudinal section through the swollen cuticle at the base of an antennal segment of an old adult (Mallory's triple stain). Shows swollen laminae made distinct by thin fuchsinophile lines, pore canals stained by fuchsin, and dark red band (*M*) separating sclerite (*S*) from the blue-staining membrane (*E*).

Figure 32. Longitudinal section of a pupal antenna that was removed from the pupal case, soaked in ammoniacal silver nitrate (1 hour), then fixed in Carnoy's fluid, sectioned and mounted without additional staining. Note the argentaffin positive granules on the surface of the cuticle and in the blood cavity within the antenna.

Figures 33–34. Longitudinal sections of the antenna of an old adult. The antenna was split longitudinally and then treated with ammoniacal silver nitrate in the same manner as the preceding. Shows dense aggregations of argentaffin-positive granules in the cells of the sense plates, and (less clearly) the absence of such from cells of the sense hairs and general epidermis.

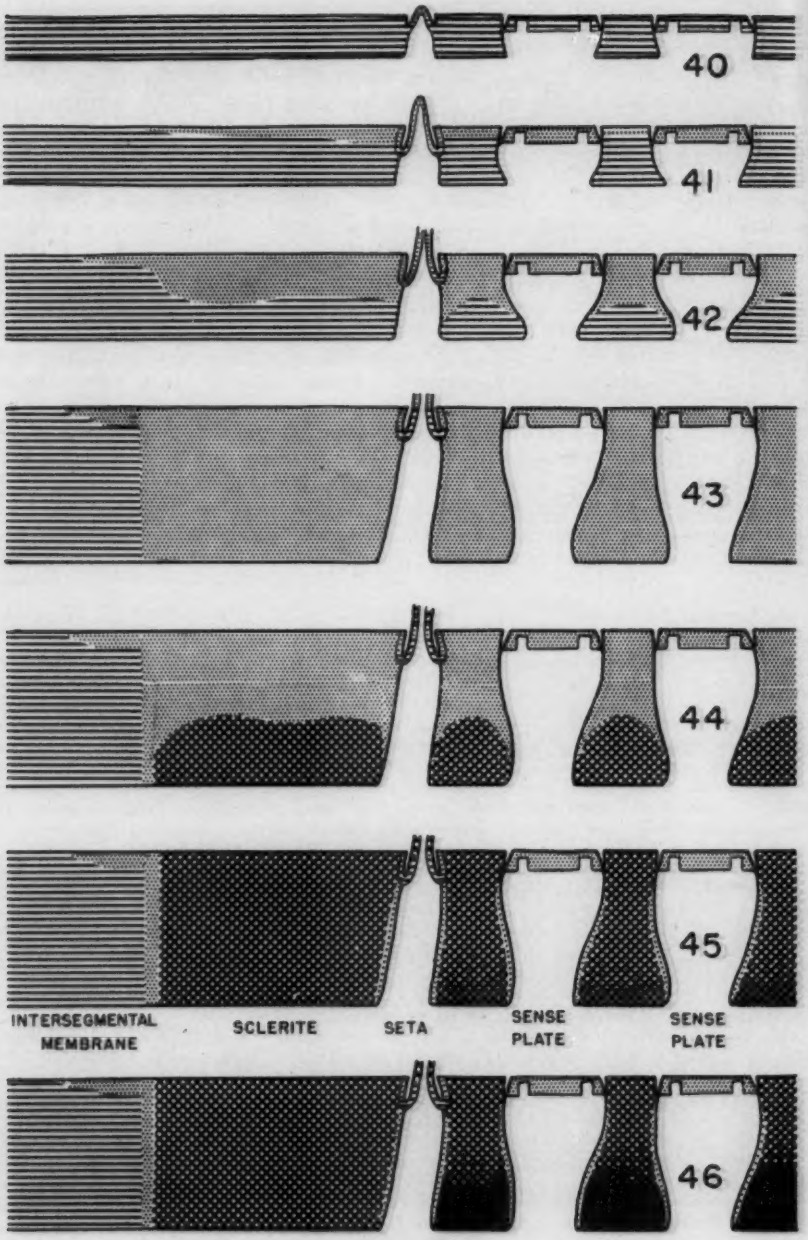
Figure 35. Cross section of the antenna of a pupa fixed in Carnoy's fluid, stained with Mallory's triple stain, and photographed with a green filter. This is an early stage in cuticle development; the print was made dark to show the precipitated molting fluid (stained blue) between the pupal cuticle (*P.C.*) and the epidermal cells (*EPID*).

Figure 36. A similarly prepared section from a slightly older pupa. Note developing sense hairs projecting into precipitating molting fluid (*E.F.*). Corresponds to an early part of the stage diagrammed in Figure 40.

Figure 37. Another similarly prepared section from a pupa of approximately the same age as the preceding. Note the distinct extra membrane.

Figure 38. A similarly prepared section from a slightly older pupa when the adult cuticle is 1–2 μ thick. Note the displaced half of a sense plate (arrow) which is lying in surface view on top of the extra membrane. Corresponds to a late part of the stage diagrammed in Figure 40.

Figure 39. A similarly prepared section from an older pupa when the cuticle (*CUT*) is nearly half the final thickness. Corresponds to Figure 42.



FIGURES 40–46

In a fully hardened cuticle, hard exocuticle is usually separated from blue-staining endocuticle by a red-staining line or band, and hard exocuticle goes through a red-staining stage during its development. Schatz proposes that the hard colored part be called exocuticle, the transparent but fuchsin-staining part be called mesocuticle, and the transparent and still blue-staining part be called endocuticle. Incidentally, the mesocuticle was the only one of these found to stain with Heidenhain's haematoxylin and with the Millon reagent. A somewhat similar picture has recently been presented by Ito (1951) and Blower (1951). Blower has proposed the term "prosclerotin" for the substance giving the Millon reaction. In the present case a purely descriptive histological term seems desirable, and mesocuticle will be used here without implying any particular chemical meaning.

Sclerotization of the bee antennae commences when the cuticle is ¼ to ⅓ its eventual thickness, and follows the novel sequence diagrammed in Figures 40–46. At first the entire procuticle stains blue with Mallory's stain (Fig. 40). Then, beginning at the outer surface, a change commences (Fig. 41) and spreads inwardly (Figs. 16, 42–43) transforming all of the procuticle of future sclerotization areas into red-staining mesocuticle. The epicuticle is not altered in its staining reactions at this time, and still seems blue over future sclerites; setae are slightly in advance of the general cuticle; the procuticle of sense plates has reached its final staining condition by the stage shown in Figure 42 and will not change further; the future intersegmental membrane thickens without changing its staining reactions; and by the time this change of future sclerite areas to mesocuticle is complete, the cuticle has reached its full thickness. Then, and this is the novelty involved, *beginning at the inner surface and spreading outwardly* (Fig. 44), the mesocuticle becomes amber colored and refractory to staining in prospective sclerite areas, *i.e.*, becomes typical exocuticle throughout its entire thickness. Subsequent to completion of this sclerotization (Fig. 45) the inner ⅓ to ½ becomes increasingly brown and eventually quite dark (Fig. 46), but for some unknown reason this additional browning of the inner portion of the exocuticle occurs only in the exocuticle between sense plates—the exocuticle of the band free from sense plates remains unmodified.

Summarizing for the condition found in the chitin-protein portion of the cuticle of the fully developed adult (Fig. 46): The intersegmental membrane stains blue and is accordingly all endocuticle except near the sclerites where the outer portion is red-staining mesocuticle. The intersegmental membrane is separated from sclerite areas by a red line of mesocuticle (Fig. 31). The sclerotized areas are completely exocuticle except for thin red layers lining the setal and sense plate apertures, but the inner portion of areas between sense plates is modified by additional darkening. Sense hairs and sense pegs become all exocuticle except for the tormogen membrane, whereas sunken setae and sense plates remain in the mesocuticle stage and have a similarly staining tormogen membrane.

This sequence in sclerotization is readily rationalized if we assume a two-stage

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FIGURES 40–46. Diagrammatic presentation of the sequence of developmental stages of the procuticle of honeybee antennae as shown by preparations stained with Mallory's triple stain. Parallel lines = procuticle and, in later stages, endocuticle; stipple = mesocuticle; cross-hatch = exocuticle; solid black = exocuticle modified by additional dark pigment. See text for explanation.

process controlled by two agents (enzymes?), one of which is effectively located at the outer surface, the other at or beyond the inner surface. Assuming two such agents, there is no known reason why they might not have the reverse distribution to that recorded for these antennae (and this is indeed what Schatz (1952) records for the cockroach *Blatta orientalis*), or both be active from the outer surface (as seems to be generally true), or both be active from the inner surface (not yet recorded).

The distribution of granules reacting with ammoniacal silver nitrate has one interesting feature. In the earliest stages, the entire antenna is negative to the argentaffin test. Somewhat later, numerous positive granules are seen in the blood space and on the outer surface of the developing cuticle (Fig. 32). Still later, positive granules are found in the epidermal cells over the entire antenna. Then, as sclerotization is completed, these granules disappear from the epidermal cells except along the soft intersegmental membrane and in the cluster of cells under sense plates. In the adult, the cells under sense plates are loaded to various degrees with large argentophile granules (Figs. 33–34) whereas cells under the general sclerite surface and under sense hairs and sense pegs are free of such granules. The argentaffin reaction is only semispecific and correlation is not proof, but the data are consistent with the idea that such granules supply the polyphenol derivative used as substrate to change the mesocuticle to exocuticle, and that this change is controlled not by which cells are permeable to this substrate from the blood but by which cells pass it out into the cuticle. A cursory check of argentaffin reactions in developing cuticles of *Blatta, Oncopeltus, Phormia* and *Apis* thoraces suggests that this argentaffin sequence may be another peculiarity of honeybee antennae.

The diffuse dark color developed in the inner part of the exocuticle between sense plates is no staining effect. It can be seen in free-hand sections examined in water and in unstained frozen-dried preparations. It is only slightly if at all decreased in intensity by prolonged treatment with hot water (Fig. 30), a treatment which changes the cuticle from virtually isotropic to moderately birefringent. It is not visibly affected by Carnoy's fixing fluid or common solvents such as alcohol, acetone, chloroform, etc. Conceivably it might be a melanin formed from excess substrate not needed for sclerotization.

Incidentally, the fully hardened exocuticle of adult antennae is darkened or blackened by ammoniacal silver nitrate and 2% osmic acid. The darkening by OsO_4 is not prevented by previous refluxing with boiling chloroform and so must be due to some agent not removed by chloroform or destroyed by heating to 61° C.

The relatively large pore canals (Fig. 6) follow a straight or slightly wavy course from the epidermis to the epicuticle, penetrating between interstices of the crossed Balken. The pore canals are stained by ammoniacal silver nitrate, Heidenhain's haematoxylin and Mallory's stain (red); they are not stained by osmic acid or by the Schiff polysaccharide test. Even in the old adult, sections in which the epidermal cells are slightly separated from the cuticle show cytoplasmic strands extending to and seemingly into the pore canals, and electron micrographs of tangential sections which have been soaked in distilled water show the canals as holes. It follows that these canals never become plugged with cuticle. The only notable point is the curious observation that in regions thin enough to see through in sections stained with ammoniacal silver nitrate, rows of spots occur along the pore

canals at intervals agreeing with the spacing of cuticular laminae. Similar precipitation of silver spots along pore canals of *Tenebrio* cuticles was obtained by Day (1949) using a technique thought by some to be specific for ascorbic acid.

Comparison of Various Sensillum Types

The sense hairs and sense pegs differ in cuticle thickness but the cuticles of both are fully sclerotized in the adult and appear completely negative to staining by Mallory's and Heidenhain's stains, 2% osmic acid and the Schiff polysaccharide test. The tormogen membrane of each of these consists of a ring of blue-staining endocuticle (Mallory's stain) separated from the exocuticle of the setal shaft by a ring of red-staining mesocuticle and connected to a similarly red-staining rim of mesocuticle.

The sense plates and sunken setae (both long and short) differ in that they stain red with Mallory's, black with Heidenhain's and pink with the Schiff polysaccharide test; as far as can be determined with the light microscope they are negative to osmic acid (see preceding section on sense plate epicuticle). These stain completely red with Mallory's, *i.e.*, unlike the sense hairs and sense pegs, they have no ring of blue-staining endocuticle.

In the pupa, the sense pegs become sclerotized first, sense hairs next, and sense plates and sunken setae last, but the time difference is not great and there is no reason to think it important since all reach whatever stage of sclerotization they are going to develop considerably prior to emergence of the adult. The real difference is that the hairs and pegs become completely sclerotized whereas the plates and sunken setae undergo only the first change (to mesocuticle). And since these changes occur early in relation to general sclerotization of the antennae, control must be somehow effected by the individual cells responsible for the formation of the various areas.

Incidentally, the tip of sunken setae may possibly be different from the shaft. At least in some slides, the tips of longer sunken setae show a minute spot of osmium deposit, or in Mallory's preparations, a corresponding blue spot. In some the tip even appears flared. Unfortunately the size of this spot at the tip of long sunken setae is close to the limits of vision with a light microscope.

The above notes comparing the various sensilla probably refer exclusively to the chitin-protein cuticle. Except for the sense plates (preceding section), details concerning the epicuticle could not be satisfactorily determined because of decreased visibility due to the cylindrical structure.

Comparison to Thoracic and Abdominal Cuticles

The preceding sections have dealt exclusively with the antennal cuticle. When this work was nearing completion, cursory examination was made to see if the novel features shown by antennae were peculiar to the antenna or just characteristic of honeybees. Only cuticles of teneral adult honeybees were examined.

The epicuticle of sclerites is negative to Heidenhain's haematoxylin. On the thorax it was negative to Mallory's stain and the Schiff polysaccharide test, but on the abdomen faint red staining was obtained on the tergites and stronger staining (especially with Mallory's) on the intersegmental membranes. It follows that

at least some areal differentiation of the epicuticle is found on other parts of the body besides the antennae but details remain to be worked out carefully. This is to be expected from the report of such areal differentiation in various species of insects (Richards, 1952b).

Most attention was given to the sclerotization picture in the chitin-protein subdivision. This clearly is not the same as on the antennae. On the thorax an outer exocuticle consists of a thin brown layer underlain by a broader amber zone; beneath this is a rather broad mesocuticle, and beneath this a thin endocuticle. On the abdominal tergum, endocuticle is limited to the intersegmental membranes but the sclerites consist of a more or less broad layer of amber exocuticle underlain by a somewhat thinner layer of mesocuticle. Adjacent to the intersegmental membrane are areas with microtrichia where the entire procuticle has been changed but only to mesocuticle.

Sclerites on the thorax and abdomen, then, present the orthodox differentiation picture. The pupal cuticle also shows the orthodox exo-, meso-, endocuticle sequence from outside to inside in sclerotized areas. These data show that the sclerotization sequence diagrammed in Figures 40–46 is a peculiarity of the antennae in honeybees, and on the antennae is shown by only the adult cuticle.

Summary

1. The epicuticle on honeybee antennae differentiates in correlation with sclerotization to give qualitatively different reactions on different parts.

2. The procuticle of the antennae shows a novel and unorthodox sequence of sclerotization involving two stages. The first stage begins at the outer surface and spreads inwardly, the second begins at the inner surface and spreads outwardly (Figs. 40–46). As a result the entire thickness of cuticle becomes sclerotized but still shows reducing power for osmic acid and ammoniacal silver nitrate. In contrast, the cuticle of thorax and abdomen shows the orthodox sequence of sclerotization proceeding from the outer surface inwardly.

3. Argentophile granules disappear during sclerotization from epidermal cells of areas that undergo sclerotization. Large numbers of argentaffin-positive granules remain in the cell cluster under the sense plates.

4. In the strip containing sense plates, the antennal cuticle develops a dark brown color in its inner half. This browning, which is additional to the usual sclerotization, is not found in the strip which is devoid of sense plates.

5. The cuticle over sense hairs and sense pegs becomes completely sclerotized; the cuticle over sense plates and sunken setae undergoes only the first of the two stages involved in sclerotization.

6. It follows that the cuticle over chemoreceptors cannot be assumed to have the same penetration properties as cuticle on other parts of the body. Also, different sensilla with cuticles of similar thickness cannot be assumed to have similar penetration properties.

7. The color of sclerotized antennal cuticle is not noticeably affected by prolonged treatment with hot water (100° C.) but lack of complete resistance to the treatment is shown by a change from near isotropic to readily recognizable birefringence.

8. The sense plates are differentiated early in the formation of the adult cuticle. They develop a complicated micelle pattern involving both radial and tangential orientations (Fig. 4). Other sensilla also differentiate early.

9. Balken are divisible into smaller fibrils of micellar dimensions. Viewed from the surface they form a crossed-fiber pattern on the antenna with four recognizable orientations: longitudinal, circumferential and two oblique (Fig. 1). At the ends of the segments these Balken fuse into rims which show completely circumferential orientation; the rims in turn join intersegmental membranes which show completely longitudinal orientation.

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THE DEVELOPMENTAL HISTORY OF AMAROECIUM CONSTELLATUM. III. METAMORPHOSIS

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A description of metamorphosis in the compound ascidian, *Amaroecium constellatum*, is presented as the third in a series of papers covering its complete life history. The paper satisfies two needs. First, it helps to fill a gap in information about a tunicate widely distributed and well represented along the eastern shore. Second, it serves as a frame of reference for the presentation of the results of an experimental study on metamorphosis which forms the subject of another paper.

The extensive studies of Brien (1929, 1930), Brien and Blanjean (1939) on the embryology and metamorphosis of European ascidians and the excellent synthesis of Berrill (Tunicata, 1950), which reviews his own investigations and those of the earlier workers, provide a comprehensive background for the understanding of the process of metamorphosis in general. Such a background precludes the introduction of unnecessary details where the processes in Amaroecium parallel those already described in the other forms.

Absence in the literature of the complete life history of any one American tunicate is justification in itself for the completion of the present study.

Materials and Methods

Amaroecium constellatum was collected in the vicinity of Woods Hole, Massachusetts. The colonies were kept in shallow dishes in running sea water and put into a dark room at night to prevent the shedding of tadpoles shortly after sunrise. About twenty minutes after removing the dishes into the light tadpoles were shed and collected into Syracuse watch glasses for study. They were studied in the living state but opacity of body necessitated extensive use of stained and cleared specimens in whole mounts and serial sections.

The fixative that gave the best results was Schaudinn's solution heated to 60° C. and poured quickly and generously over the tilted dishes to insure the greatest possible extension of the animals. The Feulgen technique used on whole mounts produced very clear specimens at all stages, even those of the early periods that remain opaque in other stains. The sections were stained with alum haematoxylin and triosin. The photomicrographs were taken with a Zeiss photomicrographic camera.

Observations

Fixation of the larva

The tadpoles swim vigorously for a few minutes after being released through the cloacal aperture of the colonies. They alternately swim and rest in short periods, being attracted first to the light side of the dish where they rest, then swim-

ming back to the side of the dish opposite the source of light (Grave, 1921). They swim for a time varying from a few to thirty minutes. At the end of the swimming period they leave the rim of water in the larger dishes and attach themselves to the side or bottom of the dish. In the Syracuse watch glasses they attach to the bottom, to the sides or to the surface film. Those that are attached to the surface film are lost when the watch glasses are put into running sea water.

At the moment of fixation the three adhesive papillae expel their secretion in an explosive action, and the attachment effected by this cementing substance may be broken without impairing a subsequent attachment at some other place. Upon explosion of the adhesive secretion the test vesicles detach themselves from the epidermal ridge, their point of origin, and move out into the tunic where they begin

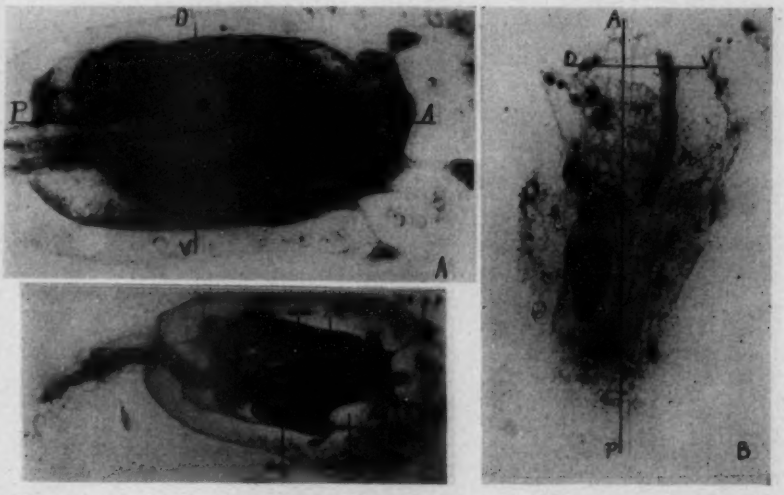

FIGURE 1. A. Tadpole at the moment of attachment showing larval axes. 200×. B. Ascidiozooid at the completion of metamorphosis, two days after fixation, showing adult axes. 50×. C. Tadpole, retraction of tail in progress. 125×. a.p., adhesive papillae; a.s., atrial siphon; end., endostyle; dig.l., digestive loop; o.s., oral or buccal siphon; per., pericardium; t.v., test vesicle; y.m., yolk mass.

to secrete additional tunicin. Within three minutes they are detached from the mantle and are distributed throughout the area of fixation. Withdrawal of the papillae from the surface and the additional tunic secreted between them produce a temporary scalloped appearance at the larval anterior end of the fixing ascidiozooid (Fig. 3, G). Since there is no addition to the test at its posterior half at this time the tunic flares broadly at its anterior half. The test vesicles rapidly lose their secretory capacity and wander out to the periphery where, eventually, they lose their identity. They function only during the period of fixation and they provide, with some questionable aid from the secretion of the adhesive papillae, a cementing substance in which the fixing larva can anchor itself during the establishment of its adult axis.

The beginning of the fixation process is marked also by retraction of the axial structures in the tail. When the tadpole comes to rest for attachment the papillae explode their contents, the tail stops twitching and, in a quick turn, arches itself into a final bend (Fig. 1, C). Within two minutes the contents of the caudal envelope buckle into one or two folds without disrupting the linear relationship of the notochord, neural tube and muscle cells. Simultaneously, the axial structures of the tail begin to retreat with smooth, flowing action into the posterior clear area of the tadpole's trunk where they are drawn into a compact mass (Fig. 3). When the caudal contents are pouring into the trunk, the cuticle becomes crinkled, giving support to Berrill's opinion (Berrill, 1947) that shrinkage of epidermis due to nutritional exhaustion may be the mechanical force initiating tail resorption. Each kind of cell retains its histological integrity and relationship during the first day and a half of metamorphosis. There is no chemical change until the differen-

TABLE I

Time schedule of rotation

Time in minutes	Tail activity	Caudal structures	Caudal sheath	Adhesive papillae	Axis of fixation	Test vesicles	Rotation
0	Contractions stop			Explosion of adhesive jelly			
1	Flexure	Buckling				Migration	
2		Beginning of resorption		Rounded knobs retreated from surface			
3			Crinkling			Distribution through area of attachment	Body contractions begun
6		End of retraction. Epidermal knob at surface	Empty sheath attached to body	Stalk detached at base or at cup by force of body contractions		Located at surface of flared tunic	
8–10		Epidermal knob within trunk			Siphonal area twisted into upright position on base of attachment	Loss of identity	Strong rhythmic contractions in progress

tiating processes of metamorphosis are accomplished. The epidermal cells at the tip of the tail together with the caudal mesenchyme form a dense cellular knob which plugs up the point of entrance of the resorbed mass (Fig. 2, A). It gradually moves away from the surface of the tunic as the caudal organs contract into their final disposition in the trunk. The empty envelope of the tail hangs on to the young ascidiozooid for two or three days and then falls off.

Withdrawal of the tail and concurrent activities of related parts can best be summarized in a chart with time schedule included (Table I).

In many cases the tail is not resorbed. It continues to hang on the body during metamorphosis executing occasional quivers up to the second and third day of development (Fig. 4, B). Action of the test vesicles and the initiation of rotation movements are the same whether the caudal elements are retracted or retained in the attached tail. Nothing in the larval structures of the tail influences the meta-

morphosic process. The enzymatic action required for autolysis or phagocytosis of the tail tissues is in no sense responsible for the activities that initiate, favor, or effect the shift in axis from larval to adult state or for the differentiation of the adult organization.

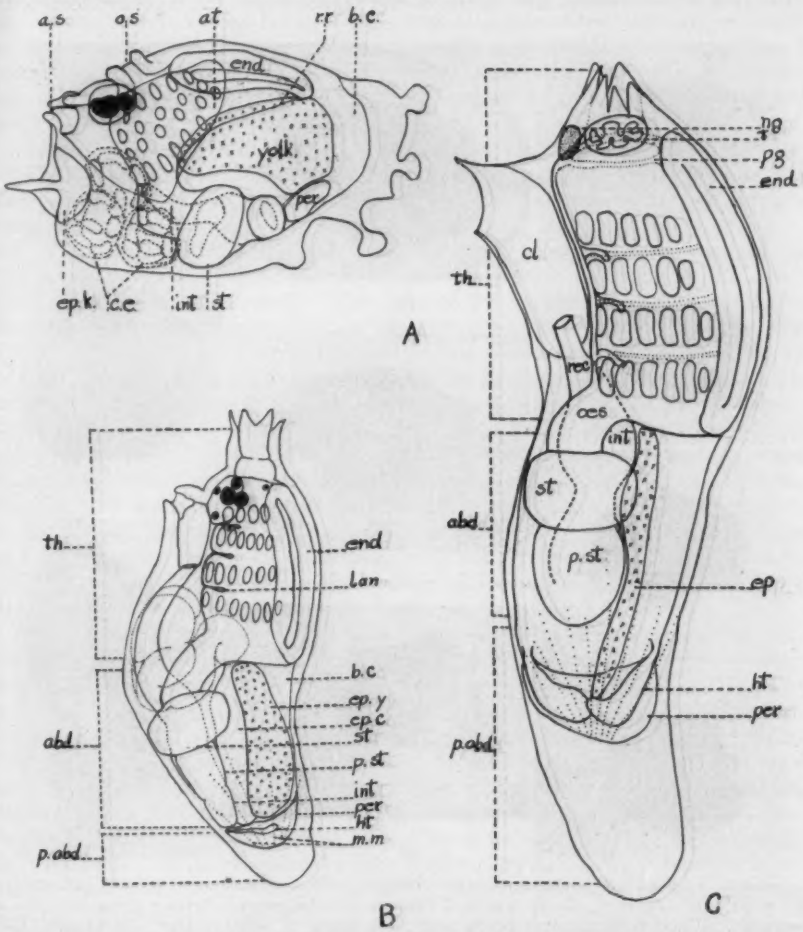

FIGURE 2. A. Tadpole at the beginning of metamorphosis. B. Ascidiozooid, 12 hours after fixation. C. Ascidiozooid during the third day of development. Abd., abdomen; a.s., atrial siphon; at., atrium; b.c., body cavity; c.e., caudal elements; cl., cloacal chamber; end., endostyle; ep., epicardial tube; ep.c., epicardial cavity; ep.y., epicardial yolk; ep.k., epithelial knob; ht., heart; int., intestine; lan., languet; m.m., muscles of mantle; n.g., neural ganglion; oes., oesophagus; o.s., oral siphon; p.abd., post-abdomen; per., pericardium; p.g., peripharyngeal groove; p.st., post-stomach; rec., rectum; r.r., retropharyngeal raphe; st., stomach; th., thorax. (*Camera lucida* drawings.)

Rotation

For clarification of terms used in ascidian metamorphosis it should be recalled that the antero-posterior axis of an Amaroecium larva is marked by the adhesive papillae at its anterior limit and base of the tail at its posterior limit; dorsal refers to the area of the siphons and ventral to the region where the digestive loop is lo-

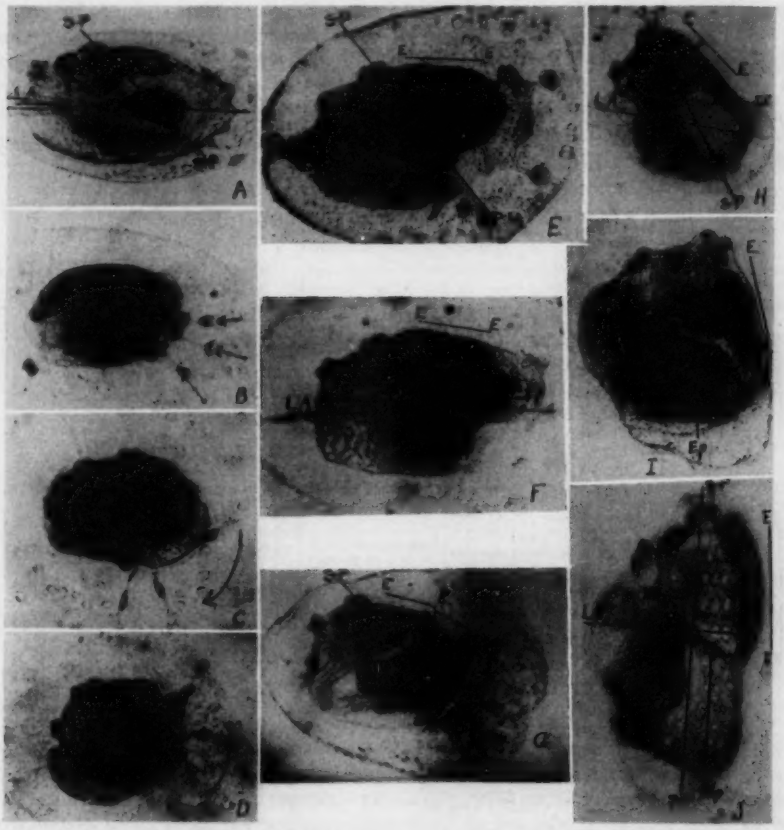

FIGURE 3. Series of photomicrographs showing body contractions of tadpole and rotation into adult axis. A, B, C, D, E, within 3 minutes of attachment. Arrows show direction of movement. F and G, 8 minutes; H, ½ hour; I, 2 hours; J, 3 hours after attachment. E–E, endostylar axis; Ep.–Ep., epicardial axis; La., larval axis; S–P, siphonal-pericardial axis; r.r., retropharyngeal raphe. About 100 ×.

cated (Figs. 1A, 2A). The antero-posterior axis of the adult Aplousobranchiata extends from the free siphonal area to the attached basal point (Figs. 1B; 2B, C). The pericardium is lodged in the basal tip of the post-abdomen. In the process of reorientation the yolk organ is projected into the lengthened post-abdomen and

the pericardium becomes separated from the digestive loop without changing their original topographical relationship (Fig. 4D). It is clear, then, that the antero-posterior axis of the adult corresponds with the dorso-ventral axis of the larva whereas the larval antero-posterior axis is temporary. The dorsal region of the adult is associated with the position of the ganglion and, therefore, all organs on that side are referred to as being dorsal; ventral is associated with the endostyle and all organs on that side are referred to as being ventral. An examination of Figures 1C and 2A will afford adequate review of the structure of the free-swimming tadpole and relationships of the larval organs to provide a basis for the presentation of metamorphic activities.

The process of rotation begins from one to eight minutes after the explosion of the adhesive jelly and the detachment of the test vesicles. It is effected by a series of contractions by means of which the organs are rotated through an arc of about 110°.

A strong contraction moves around the anterior end of the larva from the tip of the endostyle to the base of the tail on the ventral side, involving all the structures in that region of the body. The force of the movement pulls the stalks of the papillae into a sharp curve, in most cases detaching the entire papilla from the surface and reducing it to a knob-like projection of the body wall (Fig. 3A, B, C, D); in other cases jerking the stalk away from the secretory cup which is left temporarily at the surface. At the height of contraction the knobs or stalks protrude from the ventral body wall (Fig. 3C). The digestive loop is telescoped into an irregularly spiraled mass pressed against the caudal elements; the endostyle and yolk organ are shifted from the position of forming a straight angle with the siphons, coinciding with the larval axis, toward the direction of forming a right angle with the siphons and coinciding with the adult axis (Fig. 3). The endostyle may be thrown into several deep folds in keeping with the general state of contraction in all the viscera. In the living animal nothing of its anatomy is discernible when the contraction is at its maximum.

The reverse action toward the dorsal side follows immediately upon the preceding movement but it is less forceful, the organs not recovering their former alignment. Movements, once begun, alternate rhythmically through a period varying from 6 to 12 minutes, the ventral contraction always being the more violent. After that period the contractions are less frequent and less marked, each one effecting a change in the direction of the adult axis. Since the siphons and caudal mass do not participate actively in the contractions and remain unchanged in actual position and in relationship to each other in the course of the movements, it is possible to consider the siphonal area as a fixed point with reference to the reorientation of the axis. The angle of total body rotation is about 70° from larval to adult axis; the endostyle moves through an arc of 90° to 100°; the pericardium and yolk organ through 90° (Fig. 3).

The adult axis is established within two hours or less after the first contractions begin. The activities that accompany the forceful movements of rotation are processes of growth and extension, the former being marked by numerous mitoses evident in the endostyle, digestive tract and cells of the body cavity. The siphons become more elevated and prominent, the oral siphon developing six blunt lobes (Fig. 4C). Both openings are filled with primary tunic and are non-functional. The tight coil of caudal elements bulges from the surface on the morphological dorsal

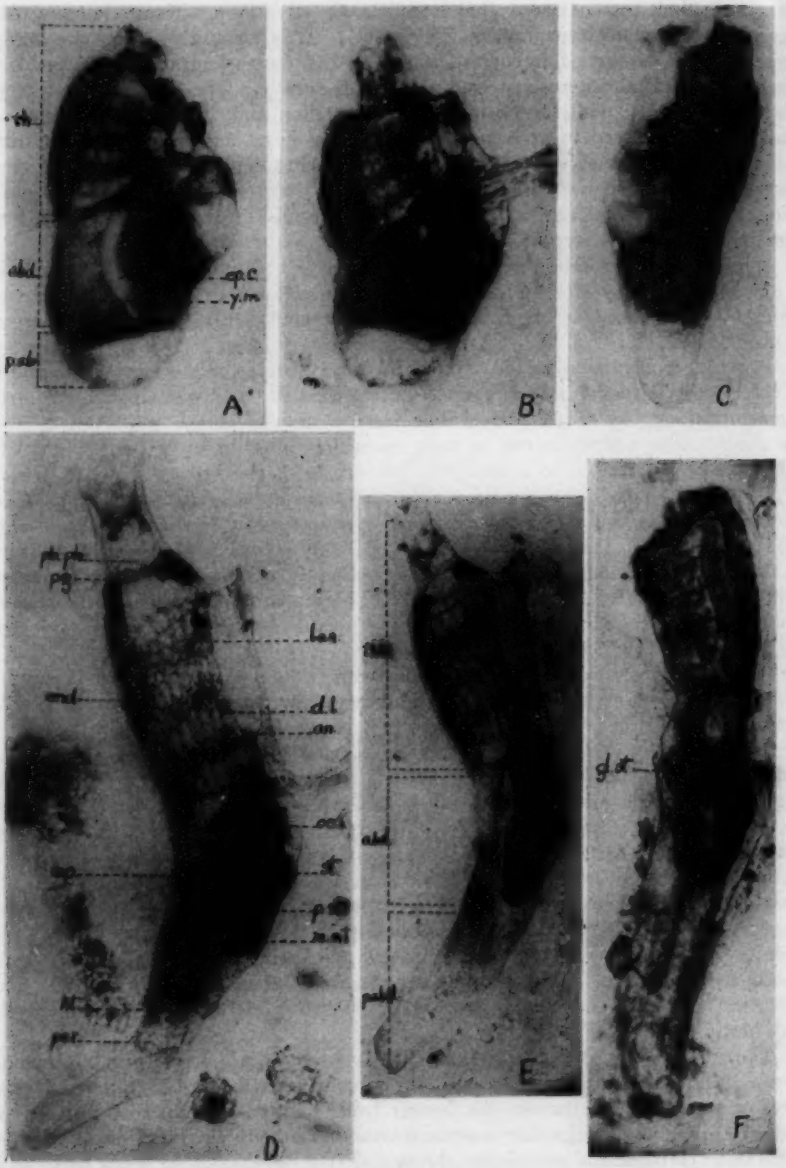

FIGURE 4

side with the intestine pressed against it medially (Fig. 4A). The organism appears short and compact during the early stages of metamorphosis and becomes progressively more transparent as reorganization proceeds.

When rotation begins, the pharynx is limited in size, the peripharyngeal cavities pressing into it laterally and the nutritive endoderm occupying most of its space ventrally (Fig. 5A, B, C). With each movement of rotation the pharynx elongates in the direction of the developing axis and the peripharyngeal cavities, correspondingly, stretch into narrow chambers clasping right and left sides of the pharynx and meeting in the common cloacal cavity at the atrial siphon. The four rows of gill clefts come into evident view as the thorax elongates (Fig. 3G, I).[1]

Expansion of the pharynx is accomplished both by extension in length in the adult axis and by gradual expulsion of the yolk organ from its location in the floor of the cavity. The yolk organ is the mass of nutritive endoderm derived from the vegetal cells. It forms two thick-walled longitudinal furrows with an extension of the pharyngeal cavity below the yolk mass (Fig. 5G). The cavity is evident as a space between digestive loop and yolk in lateral views of the tadpole. In such views the whole mass appears wedge-shaped, its broad base facing the adhesive papillae, its narrow apex pointing toward the root of the tail (Figs. 1C, 3G).

A thin epithelium covers the surface of the yolk mass and is continuous with the walls of the pharynx. The epithelium of the upper surface from the tip of the endostyle to the oesophageal opening is called the retro-pharyngeal raphe (Brien, 1929) (Figs. 2A, 3G). With each contraction the raphe is shortened and the yolk organ is gradually eliminated from its larval association with the pharynx to a position posterior to it, the two assuming a linear relationship with no communication between them. Once the separation is effected, the structure can be called the epicardium. It continues to lie parallel with the digestive loop. The yolk organ in Amaroecium has, therefore, the same significance it has in the other Aplousobranchiata, so far described, where it is similarly transformed directly into the epicardial tube.

The heart and its surrounding pericardial sac, being located at the distal end of the U-shaped intestine and epicardium or yolk organ, are involved in the rotating process to the same extent as the adjoining organs. The motion of the heart is passive insofar as it remains unchanged in structure during this time but within the first hour it commences to beat rhythmically.

Three hours after the expulsion of jelly from the papillae all rearrangement of larval systems into adult organization has advanced to the state where the animal is easily recognized as a tunicate. Within five hours the body regions characteristic of tunicates are clearly marked off: the thorax with its prominent endostyle

[1] Compactness of body and pressure of atrium into pharynx are the explanation for two previous errors in reporting the number of gill clefts as 3 instead of 4 (Scott, 1946).

FIGURE 4. Continuation of the series showing assumption of adult body form and differentiation of adult organization. A, 5 hours; B, 12 hours; C, 18 hours; D, 24 hours; E, second day; F, third day after fixation. A, B, C, 150 ×; D, 130 ×; E, F, 60 ×. Animal has begun to take food in E, second day. Abd., abdomen; an., anus; d.l., dorsal languet; end., endostyle; ep.c., epicardial cavity; ep., epicardial tube; gl.st., glandular stomach; ht., heart; lan., languet; m.int., mid-intestine; oes., oesophagus; p.abd., post-abdomen; per., pericardium; p.g., peripharyngeal groove; pb.ph., prebranchial pharynx; p.st., post-stomach; th., thorax; y.m., yolk mass.

and four horizontal rows of clefts; the abdomen lodging the epicardium and U-shaped digestive tract; the post-abdomen, in rudiment, but containing the crescent-shaped pericardium curved around the distal end of the blunt epicardium. The larval sensory vesicle is still evident. The tail structures are unchanged and protrude from the wall of the abdomen (Figs. 3, 4).

Throughout the period of rotation and beginning about fifteen minutes after fixation, mesenchyme cells of the body cavity migrate through the body wall and pass into the tunic. They are stellate in shape and produce long fibrils which extend from one cell to another. They move slowly towards the periphery of the tunic which continues to form in advance of them. The primary test of the tadpole is a secretion of the mantle. Cells continue to migrate through the body wall and they spread through the tunic.

Part of the process of reorganization includes the adaptation for adequate feeding and, therefore, freedom of movement of the oral end of the ascidiozooid. This is accomplished by flexion of the pharyngeal region on the rest of the body into an upright position so that the siphons are erected into the water. It occurs early in metamorphosis, within ten minutes, and is an action independent of the rotation movements. At the end of metamorphosis the abdomen and post-abdomen lie horizontally on the substratum to which they are attached while the thorax projects freely into the water at a right angle to the posterior body.

Further differentiation of the adult systems

When the ascidiozooid has rotated into its final axis the organs are disposed in their adult organization. The subsequent process of metamorphic differentiation is concerned with the elaboration of the systems to their mature state.

The pharynx extends into prominent occupation of the thorax. Dorsal languets project into the cavity to the left side of the points of intersection of the three horizontal branchial sinuses with the dorsal longitudinal one (Figs. 2B, 5E, F). They are similar in structure and in location in all Aplousobranchiata. The endostyle is differentiated into glandular and ciliated regions, consisting of five sections on each side. The center of the furrow is occupied by the flagellated band. On each side are the two basal glandular masses of cells separated from each other by a deep but narrow inferior ciliated band. Above the crescent shaped masses is the very slender "superior ciliated band" connecting the third glandular area with the rest and permitting it to spread widely into the floor of the pharynx (Fig. 6H). Branchial clefts number seven in each half row, tapering in size from smaller ones at both ends to larger ones in the middle.

The buccal siphon projects prominently as a rounded eminence, its collar cleft into six blunt lobes, its aperture deeply indented to the velar base which is divided into tentacles. Below the velum is the pre-branchial chamber limited by the peripharyngeal grooves into which the ciliated funnel of the hypophyseal duct enters. The peripharyngeal grooves connect the endostyle with the dorsal lamina (Figs. 2C, 4D).

The atrial siphon is less conspicuously elevated from the surface and its shallow rim remains undivided. The aperture communicates with the common or cloacal chamber of the peripharyngeal cavities. Both apertures have a layer of tunic lining

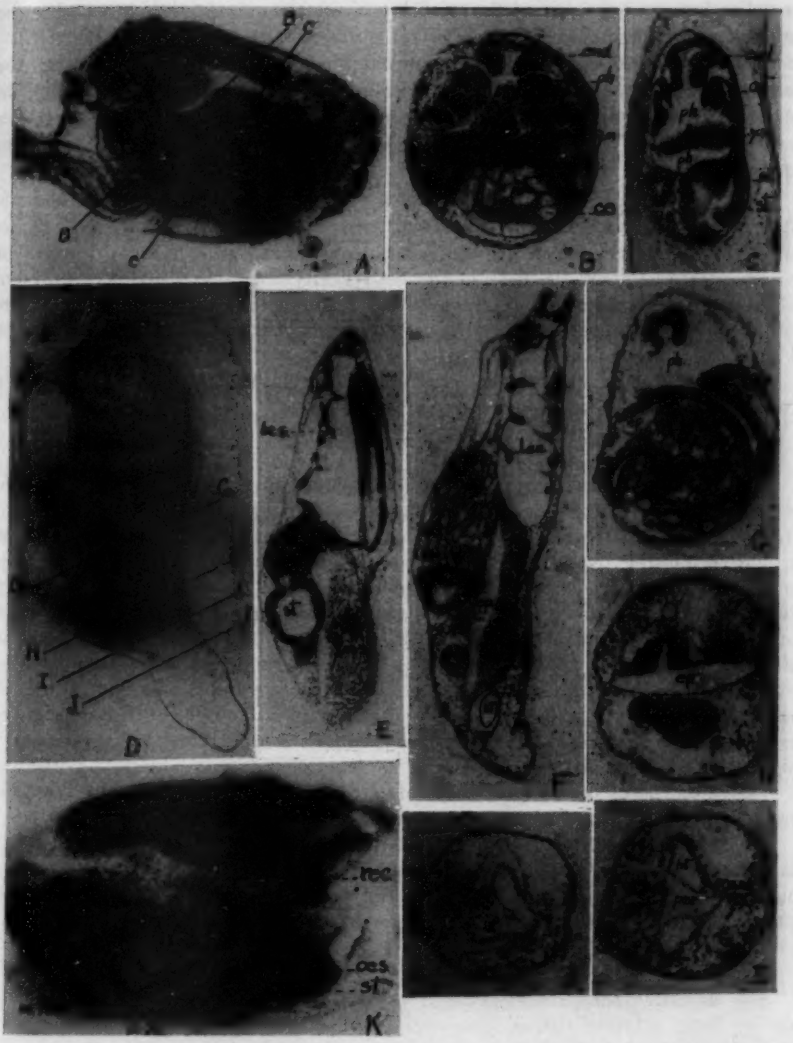

FIGURE 5. A, Whole mount of tadpole at moment of fixation with lines to locate transverse sections in B and C. D, Whole mount of ascidiozooid 12 hours after fixation showing locations of transverse sections in G, H, I and J; E and F, longitudinal sections through D; K, Whole mount of tadpole at beginning of metamorphosis showing early constriction of digestive tract. A, 200 ×; D, 150 ×; K, 250 ×. at., atrium; br.s., bronchial sinus; c.e., caudal elements; end., endostyle; ep.c., epicardial cavity; ht., heart; int., intestine; lan., languet; m.int., mid-intestine; oes., oesophagus; per., pericardium; ph., pharynx; p.st., post-stomach; rec., rectum; st., stomach; y.m., yolk mass.

the collar but until the end of the first day of metamorphosis they are both closed with a plug of tunicin.

Between the two siphons lies the neural equipment, otolith and eye in a sensory vesicle to the right, neural ganglion and subneural gland to the immediate left and visceral or larval ganglion below them connecting with the neural tube of the tail mass (Fig. 3). The latter, still unchanged, presents a prominent bulge on that side of the body.

The epicardium and digestive loop lie posterior to the pharynx in the abdomen which is not, at this time, sharply demarcated from the thorax. Both structures end at the same level. Pericardium and pulsating heart form a clear crescent immediately behind them, the area of the body cavity enclosing them being the only indication of post-abdomen present toward the middle of the first day. The three body regions of tunicates are, therefore, evident but not completely developed (Fig. 4).

The remaining period of metamorphosis may be considered under the following activities:

(1) Body growth

In the course of two days the body completes its development into the adult form. The thorax expands into a transparent urn opening through a deep collared siphon crowned with six tapering lobes. The line of the buccal orifice curves through 90° to the atrial siphon communicating with the correspondingly expanded atrial cloaca. The abdomen assumes definite regional characteristics with elongation of the intestinal loop to its full length and reduction in width of the proximal epicardium. Early in the second day, 30 hours after fixation, presence of food and associated contents in typical pellet shape makes the abdomen opaque. Continued extension of the body in the long axis produces the attenuated post-abdomen. The major length of the epicardium with its light orange deutoplasm, the pericardium and closely packed cells of the body cavity impart a faintly mottled appearance to this region. A circular rim of tissue at the level of the pericardium and distal end of epicardium holds the ends of the muscle fibres of the mantle beyond which the post-abdominal tip is completely transparent (Figs. 2 and 4).

The processes of expansion and extension are attended by mitotic activity, especially in the more compact tissues like endostyle, digestive tract, and mesenchymatous cells. Epidermal cells show scattered divisions also but the fact that the tissue is spread into a thin layer makes the figures less evident.

(2) Differentiation of the digestive tract

The digestive tract constricts into regions. The oesophagus is a broad tube curving from a funnel-shaped pharyngeal orifice to the cardiac constriction. The stomach is a short, bulbous distension, abruptly narrowed posteriorly where it continues into the post-stomach or pyloric stomach (Figs. 2, 4, 5K). During the second day of development, growth in diameter of the stomach results in the formation of about 20 longitudinal crypts characteristic of the stomach of *Amaroecium constellatum* (Figs. 4F, 6D). Histological differentiation occurs and secretion is in progress when feeding begins.

The valve at the pyloric end of the stomach opens into the short, vertical limb of mid-intestine extending to the curve of the loop where a second constriction separates it from the final division of the intestine or rectum which ascends from that point to the floor of the atrium. When feeding commences, the mid-intestine compresses the mucus-bound contents into ovoid pellets and they retain that shape until their expulsion at the anus (Figs. 4F, 6A).

Histological differentiation is confined to the organization of epithelium typical

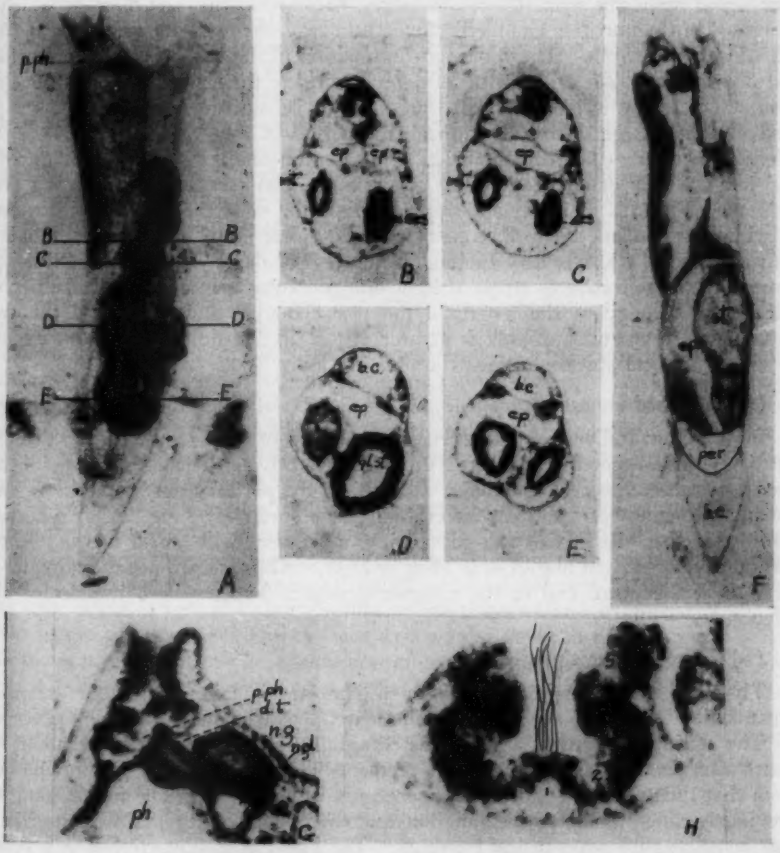

FIGURE 6. A, Whole mount of 2-day old ascidiozooid marked to locate transverse sections in B, C, D and E. F, longitudinal section through A. G, section through nervous system of adult. H, detail of endostyle with regions indicated. A, F, 100×; B, C, D, E, 150×; G, 450×; H, 1000×. B.c., body cavity; d.t., dorsal tubercle; ep., epicardium; gl.st., glandular stomach; int., intestine; m.int., mid-intestine; n.gl., neural gland; n.g., neural ganglion; oes., oesophagus; per., pericardium; ph., pharynx; p.ph., prebranchial pharynx; st., stomach.

of the various segments of the digestive tract; deep, slender columnar cells in oesophagus and mid-intestine; longitudinal glandular folds in the stomach; shallow columnar cells in the rectum. At a later stage, after budding has progressed, the pyloric gland of ascidians develops from the surface of the rectum to the pyloric stomach.

(3) The epicardium

When it is excluded from the pharyngeal cavity by shortening of the retropharyngeal raphe the epicardium is a broad, flattened cavity, the floor of which is composed of the nutritive endoderm flexed into two narrow clefts connected by thin epithelium (Fig. 5H). The roof is a continuation of the thin squamous epithelium that provides the yolk organ with its covering during the earlier stages of development. The cavity is identical with the one that becomes evident between the digestive loop and the deutoplasm during the period of rotation (Figs. 3, 4). Differentiation of the epicardium consists of elongation of the two yolk-lined furrows into long slender grooves extending through the abdomen and lengthening postabdomen (Fig. 5E, F). At anterior and posterior ends the furrows retain their individuality but lack the yolk content and the epicardium is, therefore, double at both ends (Fig. 6A–F). By the end of the second day much of the yolk is consumed but the two narrow strips of light orange deutoplasm remain to identify the epicardium throughout the length of the post-abdomen a little beyond the pericardium. The quantity of yolk is greater at the tip and decreases in amount progressively to total absence toward the pharynx (Fig. 4).

(4) Pericardium and heart

Pericardium and enclosed heart change little except in size and position throughout the period of metamorphosis. The heart is tightly constricted in its middle and opens at each end, where it remains attached to the pericardium by a narrow neck into the sub-endostylar and perivisceral sinuses respectively (Fig. 2C). The pericardium is spacious in size and curved into a crescent with distal tips of the epicardium lying at right angles to it (Fig. 5 I, J). The pericardial sac maintains its location at the distal end of the elongating post-abdomen where it pulsates conspicuously.

(5) Degenerative changes

Those organs belonging exclusively to the larval action system or "transient organization" (Berrill) are gradually eliminated by various disintegrating processes.

The sensory structures and associated ganglia begin to disintegrate about five hours after fixation, fragments of the pigment being found in any part of the body from that time until the end of metamorphosis (Figs. 4, 5). The neural ganglion, previously called the definitive ganglion, and the hypophyseal gland with its ciliated duct remain as the adult neural elements. The gland at metamorphosis is a vesicular enlargement under the ganglion, and at its posterior end. It opens into the basal chamber of the buccal siphon by a ciliated duct with a funnel-shaped aperture called the dorsal tubercle. The region of the oral siphon is referred to as the prebranchial pharynx by Berrill. It lies between the peripharyngeal bands and the velar tentacles (Fig. 6G).

The caudal mass begins to break up during the latter part of the second day. The chordal, muscle, and neural cells lose their identity and clumps of them are scattered about through the body cavity. After their disruption the body is uniformly slender except for the expanded thorax holding itself erect on the recumbent abdomen and post-abdomen (Fig. 4).

Discussion

Descriptions of metamorphosis in ascidians are confined, in general, to considerations of the morphological aspects of the reorganization of larval systems into the adult. Explanations have been proposed to account for rotation on the transverse axis in various degrees of angles from 90° to 180°. Seeliger (1885) describes in Clavelina a pocket of epidermis depressed between the papillae and the oral siphon which is everted to the surface to assist in carrying the siphons through the arc to their position opposite the point of attachment. The pocket is "spare" tissue provided during embryonic development for the lengthening of the body on that side.

Observation of metamorphosis in Amaroecium in the living state is a convincing demonstration of the active role played by contractions of the body in reorienting the animal on its axis. The muscle strands developed in the mantle of the larva effect the movements.

The larva ingests neither food nor water and, therefore, the contractions can alter the shape of the flexible organs without impairing respiratory or digestive activities. All parts of the digestive tract are involved to the extent that the enteron is literally pushed into a tortuous mass toward the oesophageal funnel. The oesophagus and oral siphon retain their relationship; the cloacal chamber of the atrium and the anus retain their relationship; the convoluted loop of stomach and intestine, at the end of the contraction period, again extends itself in length but into a straight angle with the siphon, having rotated thus through an angle of 90°.

The endostyle is a densely cellulated structure in the tadpole and plays no active role in the contractions. It may buckle into one or two sharp folds and it always becomes arched during the height of the torsion but it retains its rod-like appearance. It is moved in hinge-fashion through an arc of 90° with the siphonal region. The yolk organ is inflexible but dilation of the pharynx and extension of its wall to the length of the endostyle in the new axis presses it out of the pharyngeal cavity into the space cleared by the telescoping effect of contraction on the enteric loop. Brien (1930) refers to the exclusion of the deutoplasmic mass from the pharynx as a shortening of the retro-pharyngeal raphe in Fragarium. He does not refer to the part played by the contractions of the mantle as initiating the process but the change in relative length of the epithelium between oesophagus and tip of endostyle is the same in both ascidiozooids.

The differentiation or dedifferentiation of the epicardium from the yolk mass is similar, also, in both animals, Fragarium and Amaroecium, being a direct conversion of the nutritive yolk into the slender, yolk-retaining epicardial tube of the abdomen and post-abdomen. It may be a characteristic phase of metamorphosis in the heavy-yolked Aplousobranchiata. In general, the epicardium of ascidians is produced by evaginations of the pharyngeal floor.

During the progress of metamorphosis it is possible to see the migration of mesenchyme cells through the mantle into the tunic. They move slowly through

the tunicin until they establish a loose network with their long processes. The cells constantly change shape and the tunic becomes thicker but it is impossible to ascertain whether the cells are adding part of the secretion from their activity. Berrill (1950) is of the opinion that all tunic is secreted by the mantle or epidermis. An ânalysis of some experimental work which will follow in a subsequent paper leaves no doubt that the mantle is the seat of secretory activity early in the period of metamorphosis. This is true particularly in the area of attachment where the secretion effects adhesion to the substrate. The adhesive papillae provide for the preliminary attachment which is of transitory character, the test vesicles provide some of the cementing tunicin which is effective during the early stages of metamorphosis. Their secretory function is of short duration and is restricted to the region distal to the siphons. It seems that the test vesicles provide attachment for the contraction period when anchorage is required for the strong body movements of rotation and erection and before the mantle commences its secreting activity. The fact that the mesenchyme cells are in full march through the mantle when the tunic thickens uniformly over the surface of the ascidiozooid indicates that the final tunic may be the result of the combined activity of mantle and mesenchyme cells of the body cavity.

The points of discussion are of no controversial value but they clarify certain phases of metamorphosis in tunicates that have not been considered previously and, also, they leave another point, the origin of the tunic, to further study and analysis.

Summary

1. Metamorphosis in Amaroecium commences with the explosive release of secretion by the adhesive papillae and the steady, but rapid withdrawal of the caudal contents into a compact identifiable mass at the posterior end of the tadpole's trunk.

2. Reorientation of axes is initiated by strong contractions of the larval body which mark the beginning of reorganization in the internal organs.

3. The digestive loop, endostyle, yolk mass, and heart rotate through an arc of about 90° to their adult alignment by the action of the contractile force.

4. The pharynx expands and increases in depth being accompanied in its extension by the atrial or peripharyngeal cavities, the four rows of gill slits coming prominently into view.

5. Epicardial tube is differentiated directly from the yolk mass.

6. Typical thoracic, abdominal and post-abdominal regions of the tunicate body are developed by the end of the first twelve hours.

7. Ganglion and subneural glands are transformed into the adult nervous system while the products of disintegrating sensory pigment are scattered through the body.

8. Cytolysis of the caudal tissues begins after the adult organization has completely replaced the larval organization.

9. Reorientation of axes is accomplished within an hour after fixation of the larva; metamorphosis may be considered as completed within forty-eight hours.

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THE CONTROL OF CEMENT GLAND DEVELOPMENT IN THE CRAYFISH, CAMBARUS [1]

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The previous work concerned with elucidating possible hormonal mechanisms in the control of secondary sexual characteristics in the Crustacea has been recently reviewed by Brown (1952). That such work has to date been unsuccessful in providing indisputable evidence for an influence of endocrine secretions on these characteristics may be explained in part by reference to technical obstacles in the path of a direct application of standard endocrinological techniques to decapods. The position of the gonads in this group, together with problems imposed by the brittle, calcified exoskeleton, have rendered gonadectomy so difficult that it has not yet been successfully attempted. Takewaki and Nakamura (1944) were able to castrate an isopod, *Armadillidium*, but found no apparent influence on the development of the oöstegites in females.

The second basic problem encountered in attempting to study hormonal influences on the development of secondary sexual characteristics lies in the nature of the characters which have been considered. Those secondary characters which appear cyclically in relation to the breeding season would appear, *a priori*, to be those most readily influenced by fluctuations in hormonal balance. However, those which have been studied in this connection to date have the disadvantage of appearing as discontinuous modifications in form at the time of molt. Thus, their occurrence tends to be of an all-or-none character which renders studies of developmental rates difficult.

The cement glands of the Astacura may be considered secondary sexual characteristics on the basis of their sexually dimorphic character and the intimate relation between their discharge and the process of egg-laying. They are usually assigned the function of producing the cement which attaches the eggs to the pleopod setae of the female. Reviews of earlier literature concerning the function of these glands are provided by Broekhuysen (1936) and Yonge (1937).

Andrews (1904) described the process of egg-laying and glaire secretion in *Cambarus affinis* and stated that the cement glands in this form are chiefly found ventrally beneath the abdominal sterna and in the abdominal appendages. Farkas (1927) conducted a detailed histological study of the glands in *Astacus* and stated that the cement glands are essentially similar to the other tegumental glands described in his work. Two types of cells can be distinguished in the glands: relatively large duct cells and smaller secretory cells. The duct cells produce an intra-

[1] The author wishes to express his sincere appreciation to Dr. Frank A. Brown, Jr., for his guidance and helpful criticisms during the course of this investigation.

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cellular system of ducts which supplies the secretory cells and provides a pathway for the discharge of their mucin-like secretion.

These glands are particularly suitable for studies of possible hormonal influences on their development since their maturation occurs as a continuous process without the necessary intervention of molt. Yonge (1937) suggested the possibility of humoral control of their development but did not attempt to support this possibility. Lloyd and Yonge (1940) considered a correlation noted between the mature condition of the ovary and the cement glands in *Crangon* to be evidence for the existence of a hormone elaborated by the ovary and responsible for cement gland development. No attempt has been encountered to study possible hormonal influences on the cement glands using classical endocrinological techniques.

An important reproductive function of the crustacean sinus gland was first demonstrated for the shrimp, *Leander*, by Panouse (1943, 1944, 1946) and subsequently confirmed for *Cambarus* and *Uca* by Brown and Jones (1947, 1948). In these forms, sinus gland removal results in an increased rate of ovarian maturation. The work of Stephens (1952) provides evidence for a role of materials elaborated by the cerebral ganglia and circumesophageal connectives in the sexual cycle of *Cambarus*. Consideration of these results directed attention in the following work to the sinus gland and the central nervous system as possible sources of tropins concerned with cement gland development.

Material and Methods

The experiments to be described were performed with groups of *Cambarus rusticus* obtained from Ohio and with groups of *Cambarus virilis* collected in the vicinity of Chicago, Illinois. Reference is also made to scattered observations of other species of *Cambarus* collected locally. The protocol of each of the several experiments performed will be described subsequently.

The operative procedures were: (1) removal of both eyestalks, and (2) implantation of central nervous organs in the ventral abdominal haemocoel. The eyestalks were removed at their bases with a scalpel, thus removing the sinus glands and associated structures. The eye stubs were cauterized immediately to avoid excessive blood loss. In the case of animals receiving implants of central nervous tissues, the cerebral ganglia and circumesophageal connectives were removed from donor animals and implanted at once in a medium of van Harreveld's solution with a tuberculin syringe bearing a 16-gauge hypodermic needle. Animals were not fed in the course of any experiments.

The distribution of the cement glands will be described for female *Cambarus rusticus*. No differences in distribution were observed in other species of *Cambarus* employed. The glands underlie the second through the sixth abdominal sterna and extend laterally into the anterior region of the associated pleura. They also occur in the protopodites, endopodites and exopodites of the last five pairs of abdominal appendages. The pores through which the cement glands discharge their secretion open independently of the setae of these appendages.

In order to assess the results of experimental treatment on the development of the cement glands, a series of arbitrary stages based on their gross appearance at various points in their development has been used. Although the gross appearance as described for each stage is much the same in all of the cement glands in a

given animal, it is convenient to confine attention to the ventral surface of the uropods. The stages have been defined as follows:

Stage 1: The distinguishing characteristic of this stage is the appearance of milky-white, translucent, circular or subcircular areas, approximately 0.1 to 0.2 mm. in diameter, marking the position of the future cement gland pores. There may be 30 to 45 such areas on each ramus of a uropod depending to some extent on the size of the animal. An examination of sectioned material indicates that these areas are correlated with the appearance of the distal duct cells of the cement glands. A considerable number of ducts is associated with each presumptive pore, each duct being only about five micra in diameter.

Stage 2: The white areas described above persist and subcircular, transparent areas appear within their confines ranging from 0.05 to 0.1 mm. in diameter (Fig. 1). These transparent areas are the cement gland pores which penetrate through the chitinous layers of the integument to the non-chitinous epicuticle (Fig. 4).

Stage 3: At this stage the secretory cells have appeared in close association with the inner duct cells and are just beginning secretory activity. The glands can be seen as translucent, white, lobate clusters surrounding the cement gland pores in an irregular fashion. Each lobe represents a discrete gland, many glands providing independent ducts to one pore. Figure 3 is a photograph of a histological section of cement glands at this stage.

Stage 4: The previously described elements are still clearly visible and in addition the cement glands have become striking, opaque, cream-white as a result of engorgement with dense mucoid secretion. Figure 2 illustrates the gross appearance of cement glands which are approaching this stage. However, the cement glands eventually become so heavily charged with secretion and so distended that it is difficult to make out their lobate character. Stage 4 is the terminal or mature stage of the cement glands.

Intermediate stages of cement gland development can be defined quite accurately. Thus an animal with patent stage 1 areas plus about half the cement gland pores would be classified as stage 1.5. Such interpolations allow staging to quarter units throughout the scale. The accuracy of such determinations has been ascertained by re-reading groups of animals within an interval so brief that no significant change could have occurred. Using a group of 10 animals, the average reading checks within 0.05 unit.

One source of error was encountered in using this system. When experimental conditions are such that no cement gland development is occurring, the translucent areas surrounding or portending the pores may fade. Since they normally limn the pores, superficial examination may fail to reveal all the pores present which would produce a low reading. It was also ascertained that these areas disappear just following molt but normally reappear within 48 hours after molt.

The fading which is observed in animals which are not in immediate post-molt stages can be considered retrogressive development or it can be interpreted as a concomitant of developmental arrest. The latter alternative has been chosen in the analysis of data in this paper. Thus decrements measured in comparing stages of the same animals taken at different times are reported but are simply treated as zero increments in the calculation of averages. The alternative procedure would

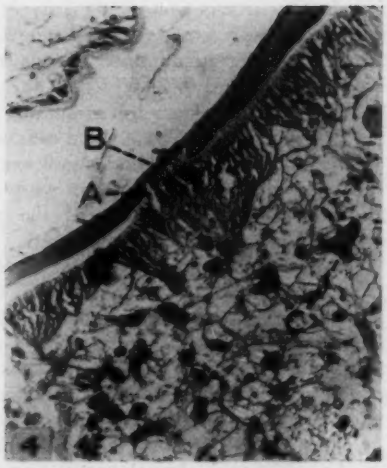

FIGURE 1. Gross appearance of the uropod of a female *Cambarus rusticus* with the cement glands at stage 2.0 (× 8).

FIGURE 2. Gross appearance of the uropod of a female *Cambarus rusticus* with the cement glands at stage 3.5 (× 8).

FIGURE 3. Histological section through uropod showing cement glands at stage 3.0. The ducts (A) can be seen lying within the masses of gland cells (B) (× 220).

FIGURE 4. Histological section through uropod showing the cement gland ducts (A) and a cement gland pore (B). Gland cells (C) which have appeared in association with the inner duct cells but have not yet begun secretion may be seen in this section (× 120).

not materially influence the conclusions suggested by the data. Animals which had molted less than two days previous to the time of a reading were not considered. Finally, it should be stressed that development as evaluated by means of the scale described above is not known to proceed at a uniform rate.

Experiments and Results

A. Influence of destalking and central nervous system implants

In the course of these experiments, it became apparent that the mature *Cambarus rusticus* females employed were responding to experimental treatment in a quite different fashion from the small *Cambarus virilis* females. It will be argued later that these *C. virilis* were immature. In any event, it is simplest to discuss the experiments employing these different species separately.

1. Cambarus rusticus

A preliminary experiment was performed over the period from July 4 to July 13, 1951. Three groups of females ranging in carapace length from 2.5 to 3.9 cm. were employed.

Group A consisted of 40 animals of average carapace length 3.26 cm., destalked on July 4. Group B consisted of 40 animals of average carapace length 3.26 cm., similarly destalked on this date and receiving one implant of nervous tissue from female *C. rusticus* donors on July 8. Group C was comprised of 15 untreated animals whose average carapace length was 3.13 cm. and served as a control group. All animals were maintained in separate compartments in running water at an average temperature of 22.6° C.

The staging system for evaluating the condition of the cement glands was not yet developed at the time these animals were isolated and consequently no values are available for the initial condition of their cement glands. Readings of each surviving animal in each group were taken on July 13, nine days after destalking and five days after implanting the groups so treated. These observations are arranged in tabular form in Table I.

A trend toward more advanced cement gland development with increasing size is apparent in all groups. However, there is considerable variation within a given size range. In an attempt to compensate for this variation, the average cement gland stage has been calculated for a series of overlapping size ranges and is presented graphically in Figure 5. This graph shows that the average development of the cement glands in those animals which were destalked and received nervous tissue implants is quite consistently more advanced than the condition manifested by the controls and those animals which were destalked but received no implants.

An additional control group was selected from the stock animals on July 13 and the cement glands staged. Although the variation between control groups was considerable, both control groups were clearly less advanced in cement gland development than the implanted animals. These results are suggestive of a possible tropic influence of the nervous system on cement gland development. In order to test this possibility further, the following experiments were performed.

Experiment 1. Two groups of female *Cambarus rusticus* ranging in carapace length from 1.7 to 2.8 cm. were considered in this experiment. Group A consisted

of 14 animals, destalked on August 22, whose average carapace length was 1.92 cm. with an average cement gland stage of 0.77. Group B consisted of 14 animals which were destalked on August 22 and received one implant of nervous tissue

TABLE I

Initial experiment with Cambarus rusticus. Condition of the cement glands of each animal in each group on July 13, 1951

Carapace length (cm.)	Destalked	Cement gland stage destalked, implanted	Control
2.5	1.5		
2.6			1.0
2.7		1.5	2.75
2.8	0.5		1.5
	1.0		1.0
2.9	1.0	1.5	1.75
	3.0	2.0	1.5
	1.25		
3.0		2.25	3.0
		1.25	
		3.0	
		3.0	
3.1	1.25	2.0	1.25
	1.5	2.0	
		1.5	
3.2	2.0	3.0	3.0
		2.0	
		1.5	
3.3	3.25	3.0	3.0
	3.0		
	3.5		
	1.5		
	1.5		
3.4	3.0	3.25	
	2.0	3.5	
	3.0		
3.5	2.0	3.5	2.0
3.6	1.5	3.0	2.75
	3.0		2.25
	3.0		
	3.5		
3.7	2.0		
3.8	3.25	1.75	3.0

from female donors on August 26. The average carapace length of the animals in this group was 2.19 cm. and their average cement gland stage was 1.14. These animals were maintained in separate compartments in running water at an average temperature of 22.5° C. The state of the cement glands was assessed on August

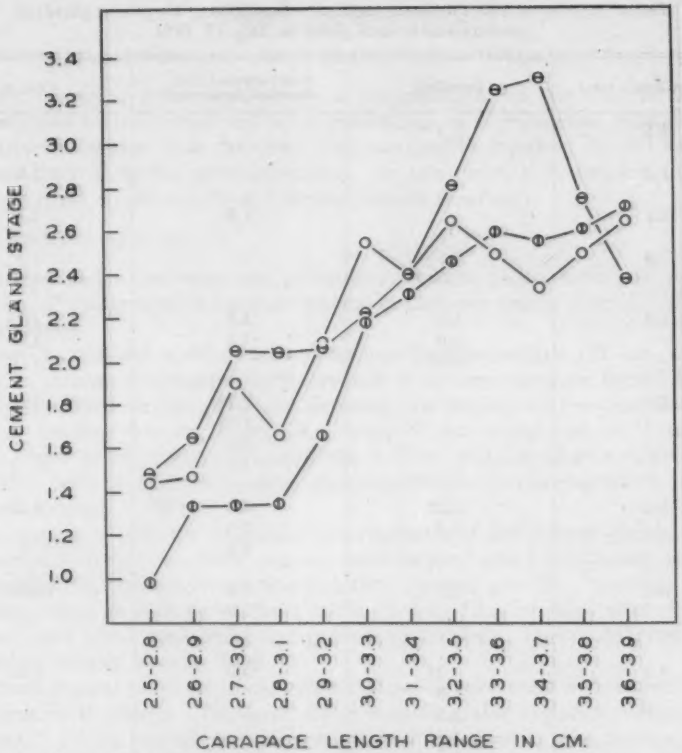

FIGURE 5. Graph showing the average cement gland stage on July 13, 1951 for the size ranges indicated of animals in groups A, B, and C of the initial experiment with *Cambarus rusticus*. Group A (destalked) ⊖. Group B (destalked, implanted) ⊖. Group C (untreated controls) ○.

26 and again on August 31. The increments for the animals in each group are tabulated in Table II. The average increment for each group was:

Group A (destalked, 9 surviving) 0.53
Group B (destalked and implanted, 9 surviving) 0.45

It should be pointed out at this time that group A in this experiment is not comparable to any other group considered because of its low initial cement gland condition.

TABLE II

Increment in cement gland stage measured over the period indicated in experiments with C. rusticus and C. virilis

Cambarus rusticus

Experiment 1		Experiment 2		
Group A (destalked)	Group B (implanted)	Group A (destalked)	Group B (implanted)	Controls (9/6 to 9/14)
1.25	0.25	0.25	1.25	0.0
0.25	1.25	0.0	1.0	0.25
1.0	0.25	0.5	0.25	0.0
0.25	0.25	0.75	0.0	0.0
0.5	0.0	0.75	1.0	0.0
0.75	0.75	0.0	1.5	0.0
0.0	1.0	0.25	1.25	0.25
0.75	0.25	0.5	1.0	−0.25
0.0	0.0	0.25	0.75	−0.25
		0.0	0.0	−0.25
				−0.25
Av. = 0.53±0.15	Av. = 0.45±0.15	Av. = 0.325±0.092	Av. = 0.80±0.17	Av. = 0.05±0.032
σ = 0.44	σ = 0.445	σ = 0.29	σ = 0.54	σ = 0.101

Cambarus virilis

Experiment 1		
Group A (destalked)	Group B (implanted)	Group C (control)
0.0	0.0	0.0
0.0	0.0	0.0
−0.25	−0.25	0.0
−0.25	−0.25	0.0
−0.25	0.25	0.0
		0.0
Av. = 0.0±.0	Av. = 0.05±0.06	0.0
		0.25
σ = .0	σ = 0.12	0.25
		0.25
		0.25
		0.25
		0.25
		0.25
		Av. = 0.125±0.036
		σ = 0.13

Experiment 2. Two groups of *Cambarus rusticus* ranging in carapace length from 1.7 to 3.2 cm. were considered in this experiment. Group A consisted of 11 animals destalked on September 20. The average carapace length of animals in this group was 1.89 cm. and their average cement gland stage was 1.66. Group B consisted of 20 females which were destalked on September 20 and received one implant of nervous tissue from female donors on September 22. The average

carapace length of this group was 2.10 cm. and their average cement gland stage was 1.63.

These animals were maintained in separate compartments in running water at an average temperature of 19.6° C. The condition of the cement glands was assessed on September 22 and again on September 28. The increments for the surviving animals in each group are tabulated in Table II. The average increment for each group was:

Group A (destalked, 10 surviving)	0.325
Group B (destalked and implanted, 10 surviving)	0.80

A control group of 14 untreated animals was maintained over the period from September 6 to September 14 to provide information concerning the normal rate of cement gland development under the laboratory conditions described. These animals were maintained in running water in separate compartments at an average temperature of 21.2° C. The condition of the cement glands was assessed on the dates mentioned above and the increments for the surviving 11 animals are tabulated in Table II. The average increment for this group was 0.05.

Before concluding the presentation of the results of these experiments, one important fact should be mentioned. The increments reported in state of cement gland development in destalked animals and in animals receiving implants after destalking are not the initial portion of a continuing increase in developmental state but rather an induced spurt of development which does not persist until the glands attain maturity. Mortality in these experiments was high and consequently observations are scattered, but no instance has been observed where induced development involved a total increment of more than 1.5 stage units. In general, no increment, or only a very small one, is measured after an initial ten-day to two-week period of response. Animals have been maintained as long as 45 days after destalking and show no substantial advance over their cement gland condition 10 days after this operation.

2. Cambarus virilis

Experiment 1. Three groups of *Cambarus virilis* females collected from a small artificial lake in the vicinity of Chicago, Illinois were employed in this experiment. Their carapace lengths ranged from 2.0 to 3.0 cm.

Group A consisted of 20 animals which were destalked on July 29. The average carapace length of the animals in this group was 2.48 cm. and their average cement gland stage was 1.44.

Group B consisted of 20 animals which were destalked on July 29 and received one implant of nervous tissue from female *C. virilis* donors on July 31. The average carapace length of the animals in this group was 2.54 cm. and their average cement gland stage was 1.46.

Group C was comprised of 20 untreated animals whose average carapace length was 2.54 cm. and whose average cement gland stage was 1.44. This group served as normal control for groups A and B.

All animals were maintained in separate compartments in running water at an average temperature of 23.0° C. The developmental condition of the cement glands was assessed on July 29 and again on August 8, 10 days after eyestalk removal and 8 days after implanting the groups so treated. The increments for the animals in

each group over this period are tabulated in Table II. The average increment for each group was:

Group A (destalked, 5 surviving)	0.00
Group B (destalked and implanted, 5 surviving)	0.05
Group C (normal controls, 14 surviving)	0.125

Experiment 2. This experiment was performed with a total of 90 locally collected female *C. virilis* ranging in carapace length from 1.9 to 2.8 cm. The animals uniformly showed cement glands at stage 2.0. They were divided into five groups as follows:

1. Destalked on November 1.
2. Destalked on November 1 and received one implant of nervous tissue from female donors on November 3.
3. Destalked on November 1 and received one implant of nervous tissue from male donors on November 3.
4. Destalked on November 1 and received an implant of muscle tissue on November 3.
5. Untreated controls.

All animals were maintained in separate compartments in running water at an average temperature of 17.0° C. The condition of the cement glands was assessed on November 1 and again on November 13. No fewer than 11 animals in each group survived this period. In no case did any animal manifest any advance in cement gland condition, all animals remaining at stage 2.0. Subsequent implantation of nervous tissue from mature *Cambarus propinquus* female donors was similarly without effect on the cement gland development of the host *C. virilis*.

The following observations seem to indicate that the animals employed in this experiment were immature. The oöcytes in these animals were small, pale, translucent white with an average maximum observed diameter (23 animals, carapace length 2.0 to 2.7 cm.) of 0.237 mm. This is to be contrasted with the presence of olive-green oöcytes of diameters ranging from 1.5 to 2.0 mm. in normal mature *C. virilis* at this time of year. In addition, the gonopods of the males of similar size and collected at the same time were uniformly of form II (the sexually inactive or juvenile form) in contrast to the form I (sexually active) gonopods manifested by mature males at this season.

Similarly the oöcytes of the *C. virilis* females employed in the first experiment with this species were small and the gonopods of comparable males were of form II. However, this is less definite evidence of their immaturity since mature females may normally have small, white oöcytes in July. However, mature males are usually in form I by the end of July so we may tentatively argue that these animals were immature. The fact that the cement glands of these animals were in stages less than 2.0 might be taken to indicate that they were hatched in the same spring. However, in the absence of observations of the cement glands of yearling, immature crayfishes, this does not necessarily follow.

B. Additional observations and experiments

Two experiments were conducted to determine if the length of the daily light period to which the animals were exposed influenced the rate of cement gland de-

TABLE III

Cement gland stages of animals subjected to 20-hour light and constant darkness, assessed on the dates indicated

Carapace length	Cement gland stage					
	20-hour light			Constant darkness		
	7/17	7/24	7/29	7/17	7/24	7/29
2.1	1.0	1.0	1.0	0.75	1.0	0.75
2.2	0.75	0.75	1.25	0.75	1.0	1.5
				1.5	1.25	
2.3	0.75	1.5	1.5			
	1.0					
2.4	1.0	1.5	1.5	1.0	1.0	1.25
	1.0	1.25	1.5	1.0	1.0	
	1.0	1.0	1.0		1.25	
2.5	1.0	1.25		1.0	1.5	1.0
				1.0	1.5	1.25
2.6	0.5	1.25	1.5	1.5	1.5	1.5
	1.5	1.25	1.5	1.0	1.25	1.5
	1.0	1.25	1.75	1.5	1.0	1.25
	1.5	1.5	1.75	1.0	1.25	1.5
				1.5		
2.7	1.0	1.0	1.25	1.5	1.5	2.0
	1.5	3.0	2.5	1.5	1.0	1.5
	1.5	1.0	2.0	1.5	1.75	1.5
	2.0	2.0	1.5	1.25	1.5	1.5
	1.25	1.25	1.75	1.25	1.25	1.75
	1.25	1.25				1.75
	1.0	2.0				
	1.25	1.5				
	1.5	1.25				
2.8	1.5	2.5	1.5	1.25	1.5	1.25
	1.5	1.75	1.5	1.0	3.0	1.5
	2.0	1.75	1.75	2.0	1.75	1.5
	1.25	1.75	1.5	1.5	2.0	1.0
			1.5	1.0	1.25	1.5
				1.5	1.75	3.0
2.9	1.5	1.5	1.75	1.5	1.5	1.5
	1.5	1.0	1.25	1.5	1.5	1.75
	1.0	2.0	2.5	2.0	2.0	2.0
			2.0			
Av.	1.23	1.49	1.60	1.30	1.45	1.52

velopment. Again there is a sharp dichotomy in the response of the mature *C. rusticus* and that of the immature *C. virilis*.

In the first experiment two groups of female *C. rusticus* were maintained in running water, one in constant darkness and the other in 20-hour daily illumination. Group A, in 20-hour light, consisted of 28 females of average carapace length 2.61 cm. and an initial average cement gland stage of 1.23. Light was provided by two 25-watt incandescent lamps suspended from a wooden frame approximately 10 inches above water level, producing an illumination of from 8 to 50 foot-candles at the surface of the water. The lights were controlled by an automatic time clock and the tank shielded with black cloth to prevent the access of undesired illumination. Group B consisted of 26 animals of average carapace length 2.63 cm. and average initial cement gland stage 1.30 and was maintained in constant darkness. The animals in these two groups were not isolated. The average water temperature over the experimental period for group A was 23.0° C. while that for group B was 22.3° C.

The cement glands of each animal in each group were assessed on July 17, July 24 and July 29. These observations are tabulated in Table III. The increments may be expressed as units per day since the two periods are not the same. The averages are:

Twenty-hour light	
July 17 to July 24	0.037
July 24 to July 29	0.017
Constant darkness	
July 17 to July 24	0.021
July 24 to July 29	0.010

The cement glands of the animals exposed to 20-hour light seem to have developed more rapidly than those of the animals maintained in darkness for both periods. In both cases, development for the first period was more rapid than for the second. Observations were discontinued on July 29 because of an increase of mortality. Since the animals were not isolated, readings of only a portion of the original sample would be of dubious value.

A second experiment of similar design was initiated on November 7 with small *C. virilis* (average carapace length 2.21 cm.) whose cement glands were uniformly at stage 2.0. It was found that alteration of daily light ration did not suffice to induce development beyond this point in any of the animals.

The extent of cement gland development was positively correlated with the size of the animals in all groups examined with the exception of groups where all of the animals were at maturity and the *Cambarus virilis* referred to above whose cement glands were uniformly at stage 2.0. Examination of groups of *C. virilis*, *C. propinquus*, *C. immunis*, *C. rusticus*, *C. diogenes* and *C. blandingii acutus* indicates that this positive correlation is widespread among crayfishes. The type of evidence obtained is illustrated by Figure 5 where cement gland stage is plotted against carapace length. *C. rusticus* provided the largest size range over which this effect could be observed. As early as July 13, large females (carapace length of 4.5 cm.) were found with typical mature cement glands while on the same date an animal of carapace length of 1.5 cm. might show no trace of these structures.

A correlation between ovarian development and cement gland development was observed in *C. rusticus*. Ovarian condition was estimated by measuring the largest observed oöcytes with an ocular micrometer and a binocular dissecting microscope and recording this size as well as the color of the oöcytes. Such measurements were

TABLE IV

Maximum observed oöcyte diameter in millimeters listed according to cement gland stage of the animals

Stage 1.0 −1.75	Stage 2.0 −2.75	Stage 3.0 −3.75	Stage 4.0
0.5	0.6	0.65	1.0
0.5	0.65	0.8	1.0
0.9	0.9	0.8	1.2
0.45	1.2	1.1	0.9
0.5	0.7	0.7	1.0
0.5	0.85	0.8	
0.7	0.7	0.9	
0.45	0.8	0.9	
0.4	0.55	0.8	
0.45	0.6	0.7	
0.55	0.8	1.0	
0.6	0.9	1.0	
0.6	0.6	0.9	
0.5	0.4	0.9	
0.45	0.9	1.0	
0.5	1.1	0.8	
0.6	0.6	0.85	
0.5	0.6	0.8	
0.45	0.65	0.9	
0.4	0.9	1.0	
0.6	0.6	0.4	
0.6	0.6	1.3	
0.7	0.7	0.5	
0.7	0.8	0.9	
0.7	0.7	0.8	
0.6	0.8	0.8	
0.7	0.7	1.0	
0.7	0.8	1.1	
0.7	0.7	0.7	
0.9	0.8	1.0	
0.6	0.7	0.3	
0.45	0.75		
0.6	0.6		
0.5	0.65		
	0.4		
Av. 0.575±0.019 σ=.114	0.720±0.028 σ=.165	0.842±0.036 σ=.202	1.02±0.049 σ=.11

made for one hundred and five *C. rusticus* females at all stages of cement gland development from stage 1.0 to stage 4.0. These measurements are grouped according to cement gland development stage and tabulated in Table IV. The average values for these groups indicate that ovarian and cement gland development proceed in a

roughly parallel fashion, though there is a considerable range of variation within each group. Similar data for other species of *Cambarus* indicate that this correlation is probably general in occurrence.

Discussion

It may be of value to provide a brief outline of events in the reproductive cycle of female crayfishes to form a basis for interpreting the results presented above. Reproductive cycles in *Cambarus* in the North Temperate Zone may be divided into two types on the basis of the time of egg-laying. Eggs are always hatched in the spring but may be laid in the fall and carried over the winter (*Cambarus immunis*, Tack, 1941), or they may be laid in the spring (*Cambarus propinquus*, van Deventer, 1937). In either case, the females present in a population in mid-summer may be divided into three groups:

1. Those animals which were mature and bore a clutch in the previous breeding season.
2. Those animals which failed to attain a minimal size necessary for reproductive activity in their first year, yearling immature females.
3. Those animals which were hatched in the preceding spring. This group can be logically subdivided into animals which will and those which will not attain the critical breeding size.

The cement glands of mature females revert to stage 0 (no gross indication of their presence) after the process of egg-laying. It is not known whether they begin to re-form immediately or there is a time lag before re-initiation of development. The cement glands of the yearling immature females attained stage 2.0 by the time of entrance into hibernaculae the preceding year. It is not known whether they continue their development from this point at the onset of spring or revert to stage 0 to re-initiate development of the cement glands. The third group of animals hatched in the preceding spring attains a stage of 2.0 or 4.0 by the following fall depending on their rate of growth and their success or failure to attain a minimal size for reproductive activity.

This cycle suggests that the correlation between size of the animals and extent of cement gland development observed during this study is not necessarily the result of a differential rate of development alone but may possibly be explained in part in terms of the three groups of animals in a population, outlined above.

Rates of cement gland development observed in unoperated animals may be listed to provide a basis for discussing variations induced by experimental operative treatment. These rates are expressed as average increments in cement gland stage per day.

Control group, *C. rusticus*	0.012
Control group, *C. virilis*	0.005
Twenty-hour light, *C. rusticus*	0.037
	0.017
Constant darkness, *C. rusticus*	0.021
	0.010

These rates may be compared with the *per diem* rates for destalked *C. rusticus* females and females receiving central nervous implants subsequent to destalking.

Experiment 1:	destalked	0.106
	destalked and implanted	0.090
Experiment 2:	destalked	0.054
	destalked and implanted	0.133

It is apparent that all of these rates clearly exceed any of the rates of cement gland development listed for unoperated animals. Thus the conclusion that destalking induces an increased rate of development of the cement glands seems justified. That this is not the result of the loss of the eyes in their capacity as photoreceptors is shown by the rate manifested by the unoperated animals in constant darkness. We may then conclude that some factor originating in the eyestalk, probably in the sinus gland, is serving to inhibit cement gland development in these animals.

The case for a central nervous tropic influence on the development of the cement glands rests on the initial exploratory experiment performed with *Cambarus rusticus* and on the results of the second experiment providing the data listed above. It was pointed out in describing experiment 1 with this species that the group of animals which was destalked was not comparable to any of the other groups considered because of the low initial stage of the cement glands. That this may have influenced the results is indicated by the fact that those animals with cement glands initially at stages less than 1.0 exhibited greater increments in this experiment than those with more advanced glands. However, although a role of central nervous neurosecretory elements in the control of cement gland development may be suggested, further experimental verification is necessary.

In no case did destalked *Cambarus virilis* females, whether receiving implants or not, exhibit significant cement gland development. The single increment measured in the implanted group of the first experiment (Table II) is within the limit of error inherent in the technique of estimation. It follows that removal of the sinus glands in these animals produced a cessation of development or failed to initiate development as contrasted with the increment in rate produced with similar treatment of *Cambarus rusticus*. Either the differences between the responses of *Cambarus rusticus* and *Cambarus virilis* to operative treatment are the result of a species difference in the controlling mechanism of cement gland development, or they are related to the fact that all of the *C. rusticus* were mature and all of the *C. virilis* were immature. In view of the close systematic relationship of the two forms, the latter explanation seems more probable.

These facts taken together suggest a controlling mechanism for cement gland development which closely parallels that postulated by Stephens (1952) for the control of ovarian development in *Cambarus virilis*. Thus the sinus gland would serve in the role of inhibitor (as shown by the increment in developmental rate in destalked, mature animals) and also elaborate a substance necessary either directly or indirectly for the development of the cement glands (as shown by the cessation of development in destalked, immature animals). The central nervous system in mature animals would serve either to store the sinus gland tropin or to convert it to an active form and store it, the rate of its release being under the control of the

sinus gland inhibitor. A system with these characteristics would explain all of the reported observations but requires further experimental support.

Callan (1940) and Knowles and Callan (1940) demonstrated the failure of secondary sexual characteristics to develop in females of *Leander* following parasitic or x-ray castration. They suggested the possibility of an ovarian hormone controlling these characters but did not rule out the possibility of a general somatic effect of such treatment. On the other hand, Takewaki and Nakamura (1944) demonstrated that surgical removal of the ovary in the isopod, *Armadillidium*, did not influence subsequent oöstegite development.

Although a correlation is reported here between the state of ovarian and cement gland development, discrepancies in this correlation actually provide evidence against the gonadal hormone theory. The presence of early stages of cement gland development concomitant with an advanced ovarian condition might be explained in terms of a failure of tissue competence by a proponent of the gonadal hormone theory. However, in Table IV, two cases are presented in the third column which show a clear discrepancy in the other direction. These animals had cement glands in stages 3.0 and 3.5 and had white oöcytes of maximum observed diameter of 0.4 and 0.3 mm., respectively. The occurrence of such animals with advanced cement glands and small, immature oöcytes would appear to argue strongly against a direct dependence of the cement glands on the ovary. It may also be pointed out that Brown and Jones (1947) report a linear increase in dry-weight of the ovaries of destalked *Cambarus immunis* over a 53-day period which contrasts sharply with the short burst of development of the cement glands followed by relative quiescence produced by similar treatment. Thus it can be tentatively concluded that the control of the cement glands, at least, is not mediated by an ovarian tropin.

Summary

1. The morphology and sequence of events in the course of normal development of the cement glands of female crayfish of the genus *Cambarus* are described and a technique for the study of the developmental rate of these glands is outlined.

2. In mature female crayfish, removal of the eyestalks produces an acceleration in the rate of development of the cement glands. Experimental evidence suggests that a further increment in rate may result when cerebral ganglia and circumesophageal connectives from mature females of the same species are implanted in the ventral haemocoel of destalked, mature animals.

3. Both destalking, and destalking with subsequent implanting of nervous tissue from immature female donors, lead to a cessation of cement gland development or fail to initiate development in immature animals.

4. The cement glands of mature females subjected to 20 hours of illumination per day appear to develop more rapidly than those of comparable animals maintained in constant darkness. However, modification of day-length is not sufficient to initiate cement gland development in quiescent immature females.

5. A correlation between developmental state of the cement glands and degree of oöcyte development is presented.

6. Characteristics of a possible humoral control of the cement glands are discussed as well as the contribution of this information to the more general subject of humoral control of secondary sexual characteristics in the decapods.

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AN ANALYSIS OF THE AGGREGATION STAGE IN THE DEVELOPMENT OF THE SLIME MOLDS, DICTYOSTELIACEAE. I. THE POPULATIONAL DISTRIBUTION OF THE CAPACITY TO INITIATE AGGREGATION [1]

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The results of a previous investigation (Sussman, 1951), indicate that the complex sequence of development displayed by the Dictyosteliaceae can occur in a population derived from a single parent cell. Moreover, the progeny of single cells, taken from widely divergent stages of growth and differentiation, retain this capacity. This would imply that, within a clonally isolated population of myxamoebae, the required cellular varieties can arise and become apparent in the necessary proportions and at the correct times so as to insure normal development. One of the interesting aspects of this implication involves the nature of the mechanisms which provide for the origin of the cellular heterogeneity observed.

The aggregation stage of the development was chosen as the subject of an initial approach to this problem. As a result of past investigation, some features of this phenomenon are well understood. A brief summary of the available information follows.

After vegetative proliferation has ceased, the myxamoebae, heretofore randomly distributed, elongate and become radially oriented. They move toward the center of orientation, at first as individuals and later in streams which become progressively larger and more ramified. In this manner, a rounded mass of cells is built up at the aggregative center. Ultimately the aggregate assumes the typical appearance of a pseudoplasmodium.

Studies by Raper (1941) indicate a complete dissociation between vegetative growth and the onset of aggregation. The results of Bonner (1947) suggest that the process occurs as the result of a response of the outlying cells to the production, by individuals at the aggregative center, of a diffusible substance or substances which Bonner has named "acrasin." Other work by Raper (1940a) indicates that the attracting substances may be specific since cell mixtures of different species will, in many cases, form entirely separate aggregation figures.

Consideration of this phenomenon suggests several pertinent questions which are capable of experimental elucidation.

(a) Is the number of aggregative centers formed a function of the number of cells in the population?

(b) If such a relationship does indeed exist, does it remain constant during the course of growth and differentiation of the population?

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(c) Is the capacity to initiate aggregation possessed by all the myxamoebae or by only a part of the population?

(d) Can a single cell initiate the formation of a center?

The present communication summarizes the salient results achieved during an attempt to answer these questions. The results indicate that the number of aggregative centers is both a function of the number of cells and of the population density. The number of centers/cell at the optimal population densities was determined for two species and was found to be constant for cells at the developmental stages immediately prior and subsequent to aggregations. Under the conditions employed the capacity to initiate center formation is not omnipresent but is possessed by only a small portion of the population. Finally, the results offer some support to the contention that aggregation centers are initiated by single cells.

Methods

A. The strains employed and their preparation for experiment

Single clone isolates of two species of Dictyosteliaceae, *D. discoideum*, strain NC-4 and *D. purpureum*, strain V-1, were employed in this investigation. Stocks were carried by plating with *Aerobacter aerogenes* on a previously specified medium (Sussman, 1951). Three to five spore masses were picked from a mature culture and mixed with a few drops of a 24–48 hour culture of *A. aerogenes*. The mixture was spread and the plates were incubated at 22° C. This inoculum requires between 44 and 50 hours to grow to maximal number and dispose of almost all of the bacteria. Subsequently, the myxamoebae aggregate and form fruiting structures.

To prepare the cells for experiment, cultures were washed off the plates with cold distilled water and the resulting suspension centrifuged five minutes at 1000–1200 rpm. in an International refrigerated centrifuge. This speed and time are sufficient to free the myxamoebae from most of the occluded bacteria. After three more washes in distilled water, the cells were suspended in either water or a salt solution devised by Bonner (1947).

B. Direct cell counts and surface density determinations

Direct counts were made with the aid of a Levy counting chamber. At least six replicate samples of each suspension were surveyed. The errors involved were found to be less than the fluctuations due to random sampling.

Measurements of surface density of cells on aggregation plates were made under low power using a Sedgewick ocular grid which had previously been calibrated with a stage micrometer. Except where later specified, the myxamoebae did not clump appreciably and the densities could be adequately controlled.

C. Medium for aggregation experiments

All studies of aggregation were carried out on plates containing a 2% washed agar-distilled water medium, hereafter designated as minimal agar. The agar was washed 5 to 10 times and dried in a vacuum desiccator before use.

Results

A. The ability of washed myxamoebae to aggregate and form fruiting structures

In his studies of the nature of the aggregative stimulus, Bonner (1947) had used myxamoebae washed free of bacteria and suspended in a salt solution. Aliquots of the suspension were dispensed in Syracuse dishes. The cells settled at the glass-water interface and, after about 16 hours, began to aggregate. Eventually, typical pseudoplasmodia were formed and at this point the morphogenetic process stopped.

For the purpose of the present investigation, a system was required which would permit accurate counts of the number of aggregative centers and precise control over population number and density. Preliminary experiments using the previously described procedure suggested that it could not completely satisfy these criteria. In devising an alternative method of study, use was made of an observation by Raper (1940b) that aggregating myxamoebae could be transferred to fresh nutrient agar plates whereupon the aggregation continued. A washed agar-distilled water substratum was substituted for the complete medium in order to suppress the growth of occluded bacteria and thus to prevent growth of the myxamoebae. Under these conditions, washed cells remained constant in number, aggregated, formed pseudoplasmodia and eventually complete and typical fruiting structures. Accurate counts of the numbers of aggregation centers were possible and adequate control over population size and density was comparatively easy to achieve.

B. The dependence of the number of aggregation centers formed upon the population size and density

Preliminary experiments were performed in which different numbers of cells were homogeneously distributed over identical areas of agar surface and, subsequent to aggregation, the number of centers counted. The results indicated a complex relationship between the number of centers and the number of cells. For very large populations, the number of centers was largely independent of the population size. For very small populations, the number of centers appeared to vary exponentially with the population size.

Clearly, a change in the total population number, distributed over a constant area must also involve a change in the population density. The possibility was raised that the number of aggregation centers produced by a group of cells is a function both of the total quantity of cells and their surface density. It therefore became necessary to elucidate the effect of the latter condition before one might hope to understand the effect of the former.

The relations between the number of centers and the population density was determined in the following way. A series of dilutions was prepared from a suspension of washed myxamoebae in Bonner's salt solution. An aliquot of each dilution was chosen such that all the aliquots contained the same number of cells. A typical protocol is given below:

No. cells/cc. of dilution	Vol. of aliquot chosen	Total no. of cells per aliquot
5×10^6	0.02 cc.	1×10^5
2×10^6	0.05 cc.	1×10^5
1×10^6	0.10 cc.	1×10^5
5×10^5	0.20 cc.	1×10^5

These aliquots were then dispensed in replicate on minimal agar plates. Since the surface area covered by an aliquot is a function of the volume delivered, the number of cells per unit area could be varied while the total population remained constant.

Trial experiments were run in order to assay the reproducibility and reliability of the procedure. It was found that the surface area covered by an aliquot is directly proportional to the volume delivered within the range employed. The number of cells/mm.^{2} of surface was determined, (a) by direct count under low power using a Sedgewick ocular grid and (b) by calculation using the known numbers

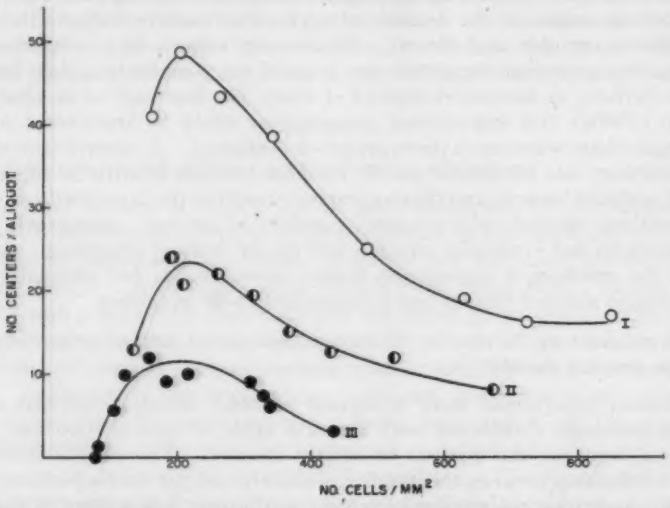

FIGURE 1. Aggregative center formation by *D. discoideum* as a function of population size and density. Aliquots of myxamoebae suspensions were placed on minimal agar plates and were designed to contain a constant number of cells dispersed at different population densities. After incubation, the numbers of centers per aliquot were determined. Curve I represents the data obtained for a population size of 1.0×10^5 cells per aliquot, curve II for 5.0×10^4 cells/aliquot, and curve III for 2.5×10^4 cells/aliquot.

of cells and measurements of the areas covered. The two procedures provided calibration data which are in excellent agreement.

After plates had been seeded in this manner, they were incubated at 22° C. with the covers ajar. Within a short time the fluid was absorbed by the agar and the covers were replaced. Determinations of population density at this time revealed no significant changes due to drying. After about 12 hours, aggregation began. Subsequently pseudoplasmodia were formed, one from each aggregation center, and these later gave rise to complete sorocarps. Counts of the numbers of centers were usually made at 16, 24 and again at 36 hours. Each aliquot was replicated between four and eight times and the means were calculated. The variance was low enough to assure the significance of the values obtained.

Figure 1 summarizes the results of a number of experiments with 44-hour cul-

tures of *D. discoideum*. The cells were harvested immediately prior to the normal occurrence of aggregation. Each point on the curves represents the mean of at least 12 replicate determinations. Three different total population levels were used.

The curves indicate that at very high population densities, the center-forming cells appear to interact competitively resulting in the suppression of a portion of them; the remaining centers are proportionately larger than those at lower population densities. At very low densities, the limit of activity of the aggregation stimulus seems to be reached so that the number of centers falls to zero. At a density of about 200 cells/mm.^{2}, no limitation is apparent and the number of centers is maximal.

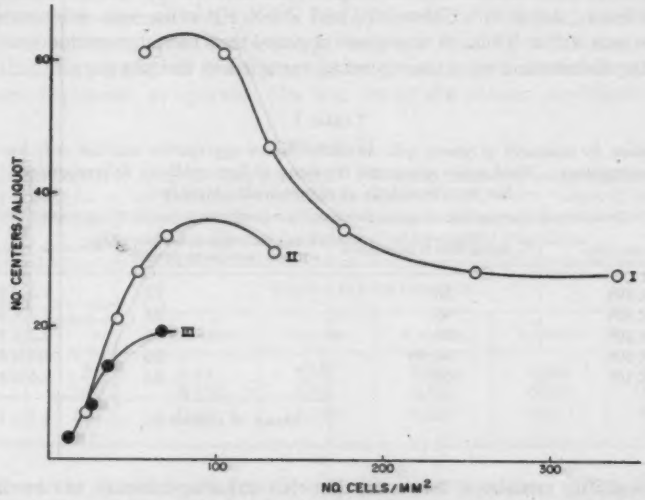

FIGURE 2. Aggregative center formation by *D. purpureum* as a function of population size and density. Aliquots of myxamoebae suspensions were placed on minimal agar plates and were designed to contain a constant total number of cells dispersed at different population densities. After incubation, the numbers of centers/aliquot were determined. Curve I represents the data obtained for a population size of 2.5×10^4 cells/aliquot, curve II for 1.0×10^4 cells/aliquot, and curve III for 5.0×10^3 cells/aliquot.

In the region of this optimal density, the number of centers was found to be directly proportional to the total number of cells present. Calculation of the number of centers/cell within this region revealed the following: for 1×10^5 cells, the number of centers/cell was 4.85×10^{-4}; for 5×10^4 cells this figure was 4.70×10^{-4}; and for 2.5×10^4 cells a value of 4.65×10^{-4} was obtained. The mean value was $4.73 \times 10^{-4} \pm 0.15 \times 10^{-4}$ (standard deviation), equivalent to one center for about 2100 cells.

A similar set of experiments was performed with washed cells from 50-hour cultures of *D. purpureum*, harvested immediately before aggregation. Figure 2 summarizes the results obtained. The number of centers/cell was found to be much higher in this species than in *D. discoideum*, the mean value amounting to

$3.3 \times 10^{-3} \pm 0.5 \times 10^{-3}$. This corresponds to a distribution of one aggregation center for about 300 cells.

C. The number of aggregation centers among cells which already have aggregated

In order to determine if the number of centers/cell was different in a population which had already aggregated, 55–60-hour cultures of *D. discoideum* were harvested, washed, and used for the experiment previously described. These cells had already aggregated or were in the process of doing so. Such cells have great tendency to clump, a condition which could be partially alleviated by the use of distilled water as the suspending medium. Even so, appreciable clumping remained and the results were somewhat erratic. The number of centers/cell for three population levels, 1.0×10^5, 5.0×10^4, and 2.5×10^4 cells, was determined. The mean value was 4.3×10^{-4}. It therefore appears that the aggregation process does not alter the distribution of center-forming capacity in the population.

TABLE I

Center formation by mixtures of young cells harvested before aggregation and old cells harvested after aggregation. Each value represents the mean of four replicate determinations. See text for details of experimental procedure

No. cells per drop	% young cells in population	Mean no. centers/drop at optimal population density	Mean no. centers per cell
1.8×10^5	50	77	4.3×10^{-4}
1.8×10^5	90	93	5.2×10^{-4}
1.8×10^5	99	94	5.2×10^{-4}
1.8×10^5	99.99	76	4.3×10^{-4}
1.8×10^5	100	82	4.6×10^{-4}
		Mean of means 84	4.7×10^{-4}

The possibility remained, however, that this value is constant not because more cells had not attained the ability to initiate center formation but rather because the remainder of the population could not respond to the stimulus imposed. In order to rule out this possibility determinations were made of the number of centers/cell of a series of mixtures of old cells (55–60 hours) and young cells (44 hours). Table I shows the results obtained. Regardless of the proportion of young and old cells, the number of centers formed did not differ beyond experimental error. The similarity was evident for each of the population densities employed.

D. The populational distribution of center-initiating capacity

Under conditions where the degree of cellular dispersion does not limit the initiation of aggregative centers, the number of centers which are formed was found to be directly proportional to the number of cells present. At least two interpretations may be raised to explain this fact.

(a) All individuals in the population are capable of initiating aggregation, acting either singly or in association. However, the realization of this potency does not occur simultaneously in all individuals but rather is distributed in time. Thus, the small proportion of cells which attained this capacity first would be the only

ones to initiate centers. The rest of the population, attaining this ability at a later time, would already have been attracted to previously established centers and so could not inform the observer of their acquisition.

(b) The formation of a center depends upon the presence in the population of a single cell or group of cells which is uniquely constituted so as to be able to initiate the formation of the center.

A distinction between the alternatives was made by examining the distribution of center-forming capacity within very small population samples at population densities previously shown to be sufficient to permit the expression of aggregating ability. Were alternative (a) valid, one might anticipate that at least one center of aggregation would appear in every sample even though the times of appearance might differ. In contrast, the validity of (b) would be evidenced by the fact that, for small enough population samples, not every one would produce a center despite the fact that the population density would be great enough to enable the initiating mechanism, if present, to operate. In fact, under the proper conditions the distri-

TABLE II

Population distribution of center-forming capacity. The mean number of centers/cell was calculated by solving for m, the first term of the Poisson expression, $P_o = e^{-m}$, where P_o is the fraction of drops containing no centers and m is the average number of centers/drop. See text for details of experimental procedure

No. cells per drop	No. drops examined	Fraction of drops containing:				Mean no. centers/cell
		No centers	1 center	2 centers	3 centers	
2100	85	0.42	0.50	0.07	0.012	4.1×10^{-4}
1025	91	0.626	0.32	0.044	0.011	4.6×10^{-4}
900	123	0.69	0.285	0.025	0.0	4.1×10^{-4}

bution of centers should be in accord with the Poisson series. Thus, the proportion of samples containing 0, 1, 2 and 3 aggregative centers would be given, respectively, by the expression, e^{-m}, me^{-m}, $m^2e^{-m}/2!$, and $m^3e^{-m}/3!$, where m is the mean number of centers per sample.

Suspensions of washed 44-hour *D. discoideum* were prepared and replicate, direct cell counts were made in the Levy chamber. A Cenco machined loop of 1.0 mm. inside diameter was used to deposit drops of the suspension on minimal agar. The fluid was quickly absorbed leaving the cells homogeneously distributed. The average volume of the drops delivered by the loop was found in trial experiments[2] to be 1.1×10^{-4} cc. $\pm 0.1 \times 10^{-4}$. Surface density measurements were made as before with a Sedgewick ocular grid and demonstrated the duplicability of the method. The variances of the determinations were in all cases approximately equal to the means, indicating that the only significant error is that incurred by random sampling.

[2] Very dilute suspensions of myxamoebae were dispensed with the loop. The mean numbers of cells deposited were determined by direct count under low power. The original suspensions were counted with a Levy chamber. From the two sets of data, the volume delivered by the loop could be calculated.

After 16, 24 and 48 hours incubation, each spot of cells on the agar was examined under a dissecting microscope for the presence of aggregations and fruiting structures. At the end of this time, it was found that not all of the spots contained centers of aggregation. Moreover, all the centers which appeared did so before 24 hours. Table II summarizes the data obtained from three experiments in which samples of 900, 1025 and 2100 cells were used. The last column on the right contains calculations of the number of centers/cell. These were obtained by solving the first term of the Poisson expression, $P_0 = e^{-m}$, where P_0 is the proportion of samples containing no centers and m is the mean number of centers per sample. The average number of centers per cell was found to be 4.3×10^{-4}. This compares well with the figure, 4.73×10^{-4}, obtained by the procedure described in Section B.

One may ask whether the suppression of center formation by high population densities might not increase the proportion of samples displaying no centers. That

TABLE III

Comparison between the observed and expected distributions of aggregative centers. See text for details

No. cells/sample	2100		1025		900	
m	0.86		0.47		0.37	
Total samples	85		91		123	
No. with 0 centers	36		57		85	
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
No. with 1 center	42	30.9	29	26.6	35	31.4
No. with 2 centers	6	13.3	4	6.2	3	5.8
No. with 3 centers	1	3.4	1	0.96	0	0.71
χ^2	45.6		1.00		2.44	
p	<0.01		0.6		0.3	

this is not the case can be seen by comparison between the data obtained with 900 cells and with 2100 cells. In the latter samples the population density was more than twice as great as in the former. Yet, despite the greater cell density, the proportion of samples displaying no centers was much smaller. It must be noted in this connection that high cell densities do not inhibit all center formation *per se*. From the results summarized in Figure 1, it would appear that when the population density is high, center initiators are close enough to compete for aggregatee cells and, as a result of this competition, some prospective centers are incorporated by their neighbors. In no case has the existence of a high population density led to the suppression of all centers but merely has acted to reduce the total number. Thus one might expect that in the small population samples at high cell densities, the presence of one initiator would lead to the production of a center but where two or more initiators were present in a single sample, the number of centers formed might be less than the number of initiators. Were this condition to exist, the P_0 values would provide accurate reflections of the distribution of initiators but the

P_1, P_2, etc., values would be biased. For this reason, only the P_0 values have been utilized in the calculation of the number of centers/cell.

It must be emphasized that the crucial point of the experiment has to do with whether or not every sample can produce a center. The question of agreement with the Poisson series is of secondary importance, for its purpose is to enable us to calculate the number of centers/cell and thus permit a comparison with the figure derived previously.

A rough estimate of the agreement of the observed data with the Poisson distribution was made in the following way. The values of m, obtained from the P_0 figures, were employed in order to calculate the corresponding P_1, P_2 and P_3 values assuming random distribution. Table III compares these figures with those obtained experimentally. The accompanying chi-square estimates indicate that, for the experiments using 900 and 1025 cells/sample, the differences between observed and expected results should be exceeded by chance 30 and 60% of the time, respectively. For 2100 cells/sample, the agreement is very poor. Here, the biasing of the P_1, P_2, etc., values, anticipated previously, was encountered. This is not surprising in view of the fact that the population density of these samples was between 500 and 600 cells/mm.^{2}.

It appears that more efficient utilization of the data might be accomplished by making use of the P_1, P_2, etc., values from the experiments where these values were not biased. However, it is felt that the precision required in order to obtain a definitive answer to the question posed does not warrant this refinement.

Discussion

The reported findings indicate that, by the end of the growth period, a clonally derived population of myxamoebae exhibits a condition of heterogeneity with respect to the ability to initiate aggregation. The question of whether this initiation is contrived by a single cell or by groups of cells acting in concert cannot be answered conclusively from the data at hand. However, two considerations point to the likelihood that the former interpretation is correct.

(a) Microscopic observation of the process reveals that at the beginning of aggregation, the cells elongate and become radially oriented. If the population density is small, it generally may be observed that the center of orientation is occupied by very few cells and often merely by one.

(b) The distribution of aggregative centers among small, replicate samples of a population indicates a constant value for the number of centers per cell despite the fact that in these experiments the mean number of cells per sample was varied and therefore the population density was not held constant. Yet, it is clear that, were a group of cells to cooperate in the initiation of a center, an increase in density would encourage center initiation. In the experiments described, however, increases in density did not affect the proportions of samples with no centers but merely acted to suppress the formation of additional centers in samples which already possessed one or more.

One possible method of distinction would appear to lie in the examination of center formation by small numbers of wild type myxamoebae when mixed with "aggregateless" mutants, capable of responding to the initiating mechanism but not

of producing it. A number of aggregateless variants have been isolated and are being investigated with this end in view.

One may inquire into the genetic mechanism by which the aggregators and aggregatees arise. Previous findings (Sussman, 1951) have indicated that the differentiation observed could equally well arise via stable genic mutation and appropriate selection pressures, segregation or zygote formation within the clone, or by easily reversible genic or cytoplasmic modifications. At present, it does not appear that the results reported here can be construed to support or militate against any of these possibilities. However, it is hoped that with the information now at hand, a fruitful examination of this question will be possible.

The relation between the center-initiating mechanism and the production of acrasin, the chemotactic principle, may be examined in the light of the reported results. Observation of the aggregative process (Bonner, 1944) has indicate that the myxamoebae at the periphery of the aggregation pattern do not in all cases move radially toward the center but often migrate toward previously established streams of aggregating amoebae before taking up a radial direction. This would indicate that the production of acrasin is not a property possessed only by individuals at the aggregating center but also can be accomplished by outlying cells. It is therefore likely that the capacity to initiate a center of aggregation is not identical with the ability to produce acrasin. One may speculate that the center-initiating agency may supply a co-factor necessary to the production of acrasin and thereby exert its effect.

Summary

1. Examination of the aggregation stage in the development of the Dictyosteliaceae has revealed that the number of aggregative centers formed is a function of the population size and density. At population densities which do not limit the formation of centers, the number of such centers is proportional to the number of myxamoebae present. For *D. discoideum*, the number of centers/cell was found to be 4.73×10^{-4} and for *D. purpureum*, 3.3×10^{-3}. These values correspond to one aggregation for about 2100 and 300 cells, respectively. The values remain constant throughout the aggregation process.

2. Analysis of the distribution of centers within small, replicate, population samples of myxamoebae indicates that the capacity for center initiation is possessed by only a small proportion of cells. The values for the number of centers/cell achieved by this method agreed well with those previously cited.

3. The implications of these findings are discussed.

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STUDIES ON SHELL FORMATION. I. MEASUREMENT OF THE RATE OF SHELL FORMATION USING Ca^{45} [1, 2]

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Shell growth is commonly measured in ecological and conservation studies as a linear increase occurring in one or a few weeks, or during a shorter period if growth is very rapid. For such studies measurements of growth increments of the order of one millimeter are usually sufficiently sensitive. For the experimental analysis of shell formation, the availability of a method of a different order of sensitivity would be distinctly advantageous and at the same time would make possible new approaches to the study of shell growth. The extreme sensitivity of radioactivity measurements suggested the use of radioisotopes. The determination of the rate at which Ca^{45} is incorporated into growing shell should give a measure of the amount of mineral substance deposited during shorter intervals than would be otherwise possible. The isotope method would also permit for the first time the quantitative measurement of mineral deposition in localized areas of the inner shell surface.

In the present series of studies the radioisotope method has been utilized in the analysis of several phases of shell formation. These include: (1) the quantitative measurement of calcium deposition; (2) rate of transfer of calcium through the mantle; (3) the distribution of inorganic and organic material deposited on the inner shell surface as studied by autoradiographic and histochemical methods; and (4) the exchange of calcium between shell and sea water in the absence of living tissues. This study deals with the first of these problems and is specifically concerned with the examination of the suitability of the isotope method for measuring calcium deposition in the living oyster under standardized conditions. These experiments demonstrate that the isotope method can be used to measure the deposition of calcium and that growth increments occurring in four hours are measureable.

Methods

Oysters (*Crassostrea virginica* Gmelin [3]) were collected from Bogue Sound near Beaufort, N. C., and kept below low tide level at the laboratory until ready for use. Those to be used for a particular experiment were matched approximately as to size (ca. 8–9 cm. in length) and the surfaces scrubbed free of foreign matter. After drying briefly they were coated with fingernail polish to prevent uptake of Ca^{45} by

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[3] Previously *Ostrea virginica*. Re-named by Gunter (1950).

the outer surface of the shell.[4] The coated oysters were kept below low tide level for several hours or overnight to remove all traces of the solvent.

Single oysters were placed in one liter of sea water in large flat glass dishes and shell movements were recorded on a kymograph throughout the experimental period. Such records provided data on periods of closure as well as indicating normalcy of shell movements. If shell movements were normal, the sea water was renewed and Ca^{45} added to give an activity of 6×10^5 counts per minute per liter of sea water. The Ca^{45} was of high specific activity or carrier-free so that the calcium content of the sea water was not increased appreciably. The radioactive sea water was renewed every four hours. The temperature range was 21.2 to 25.4° C. for the entire period of study though approximately constant for each experiment. Sea water salinity was approximately 35 parts per thousand.

After intervals of 8, 16 and 24 hours, oysters were removed from the sea water containing Ca^{45} and the shells prepared for determinations of the radioactivity of the inner shell surface. The mantle was removed and the region of muscle attachment carefully scraped free of material. The shell was then rinsed thoroughly with a fine stream of distilled water from a wash bottle. Previous experiments had shown that this treatment was sufficient to remove all adhering material which might be radioactive and was as effective as scrubbing with a stiff brush. The radioactivity was measured for circular areas of 6.2 cm.^{2} in the following regions: extreme posterior end; center which included a portion of the site of muscle attachment; and the anterior end immediately posterior to the hinge. Essentially constant geometry was maintained by means of a special holder in which the shell was held close to the window of the counting tube for the measurements just mentioned or against a brass plate with a circular aperture of 1 cm^2. for more exact localization of radioactivity. Because of geometrical difficulties with the cupped left valve, measurements were confined to the less curved right valve. A thin window counting tube (1.9 mg./cm^2.) was used.

Carefully cleaned right shells were included in all experimental solutions in order to obtain an estimate of the radioactivity of the shell resulting from exchange between sea water and shell as contrasted with deposition by the living oyster. The values for the living oyster were corrected for exchange in each instance.

Results

Figure 1 shows the Ca^{45} deposited after various intervals in the posterior region of shell of 60 oysters. The mean Ca^{45} deposition was directly proportional to the period of exposure to the isotope. Marked individual variation is apparent and will be discussed later. Graphs of the center and anterior regions gave a similar picture but differed quantitatively from the posterior region. Calcium deposition could be measured after four hours in radioactive sea water under these conditions, as suggested in Figure 1 and as demonstrated in experiments not reported here. With higher concentrations of Ca^{45}, deposition would be measurable after still shorter intervals.

The deposition rate was not uniform over the shell surface; the relative mean depositions after 24 hours for the three regions measured were: posterior 1.0, cen-

[4] P^{32} is taken up by nail polish though not by a coating of wax.

ter 0.55 and anterior 0.58. The regional differences were investigated further by making measurements of Ca^{45} activity on several circular areas of 1 cm.^{2} (Fig. 2). The positions of the columns indicate the regions for which radioactivity was determined and the height gives the relative deposition. The non-uniformity of rate of deposition measured in small areas is more marked than with the larger areas mentioned above. This general pattern, which represents the means from 8 oysters, also characterized the individual shells. The main features of the regional

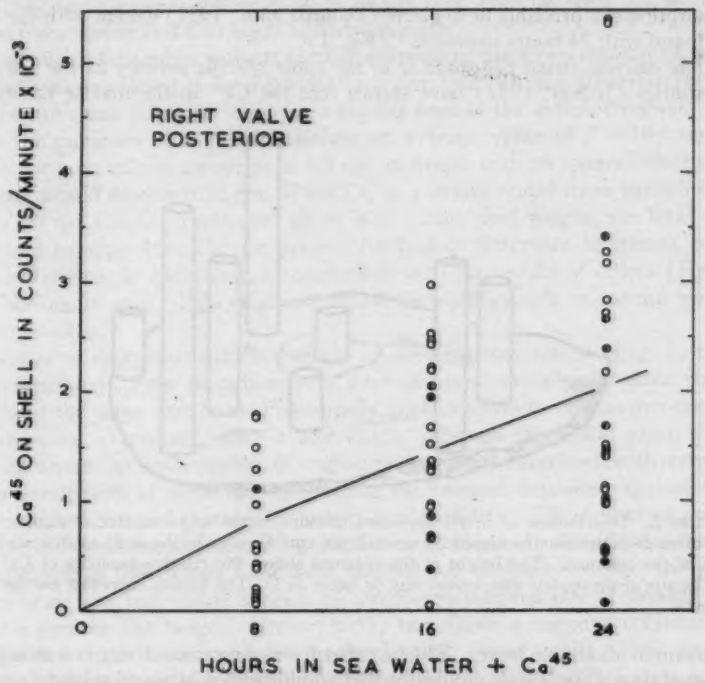

FIGURE 1. Ca^{45} deposition by the living oyster. Each point represents the radioactivity of a circular area of 6.2 cm.^{2} at the extreme posterior end on the inner surface of the right valve of one oyster. The line connects the mean values for Ca^{45} deposition for each interval. The half closed circles represent Ca^{45} deposited by oysters which remained closed for more than 25% of the experimental period.

differences are the following: (1) The differences in deposition in adjacent small areas may be great. And the pattern of rates obtained will accordingly depend upon the size of the area included in the individual measurements, as may be seen by comparing the values for the three larger areas with those in Figure 2. (2) The rate of deposition in the posterior portion was relatively high, although the thin posterior edge was low. (3) The portions near the periphery of the shell exhibited a higher rate than the interior. An autoradiographic study, which will more exactly define the pattern of deposition, is now in progress.

An approximation of the weight of calcium deposited can be made by comparing the radioactivity of the sea water and the shell. Such an estimate becomes possible when the following conditions are operative:

1. *The measured radioactivity of the shell is directly proportional to the total Ca^{45} deposited in a given interval.* This will be the case provided the layer of new material is sufficiently thin that the radiation from the calcium first laid down is not absorbed by the salts and conchiolin deposited later. The estimated amounts of $CaCO_3$ deposited (see method below) in the intervals used are such that the factor of self-absorption can probably be neglected (Comar *et al.*, 1951) except with the higher values found with 24 hours exposure [5] (Fig. 1).

2. *The calcium being deposited is of the same specific activity as the sea water.* Other studies (Jodrey, 1952) have shown that the Ca^{45} in the mantle has reached

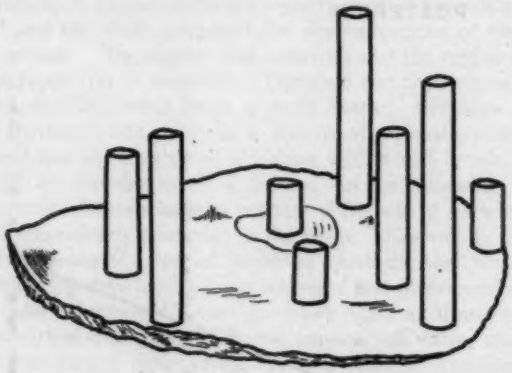

FIGURE 2. Distribution of newly deposited calcium on the inner surface of shell. Ca^{45} deposited after 24 hours was measured on several one $cm.^2$ regions of the shell as indicated by the position of the columns. The height of the columns shows the relative amounts of Ca^{45} deposited. The site of the muscle attachment may be taken as 1. The figures represent average values for 8 shells.

equilibrium in about one hour. The fact that the mean radioactivity is a straight line function of time (Fig. 1) also indicates that equilibrium is attained relatively quickly.

3. *The difference in backscatter in measurements of radioactivity of sea water and shell is known.* Radioactivity measurements of the sea water were made on copper planchets whereas measurements of shell were made against the $CaCO_3$ of the shell itself. The higher atomic number of copper as compared with calcium would give an increased backscatter and an underestimation of the calcium deposition of the order of 8 or 9% (Glendennin and Solomon, 1950).

4. *The specific activity of the sea water is not appreciably changed by unlabeled calcium from the tissues and body fluids.* Error from this cause would be negligible

[5] With the mean deposition rate of 9.0×10^{-6} mg. $CaCO_3/cm.^2/hr.$ found in the posterior region, the limiting value of approximately 0.5 $mg./cm.^2$ would be reached in about 56 hours. For the shells showing most active deposition (Fig. 1) the limiting value has already been exceeded slightly in 24 hours.

because of different orders of magnitude (ca. 400 mg. Ca per liter of sea water and ca. 7 mg. Ca in the oyster (Sherman, 1947)). The renewal of the radioactive sea water every four hours would further minimize this factor.

Under the conditions defined above the calcium deposition can be calculated from the following relation:

$$D = \frac{A_s}{A_w} \times C,$$

where D is mg. Ca deposited cm.^{2}, A_s is counts/min./cm.^{2} on shell, A_w is counts/min./liter sea water, and C is mg. Ca/liter sea water.

Using this relationship without further correction, the mean amount of calcium deposited was calculated to be 9.0×10^{-3} mg./cm.^{2}/hr. for the posterior region of the right valve and 5.2×10^{-3} mg. for a similar area in the anterior region. If we assume for purposes of a rough estimate an average value of 7×10^{-3} mg. Ca/cm.^{2}/hr. for both valves, an oyster of 8.5 cm. in length with an internal shell surface of 70 cm.^{2} would deposit 0.92 gm. of $CaCO_3$ in a month under these particular conditions. If the $CaCO_3$ constituted about 90% of the shell weight, the total weight gain would be approximately one gram. Because of difference in species and unknown differences in conditions, a comparison with the results of others (Havinga, 1928; Masumoto *et al.*, 1934; Galtsoff, 1934) on shell growth in natural waters is scarcely possible.

The rate of deposition differed widely in different individuals (Fig. 1) as previously mentioned, even though oysters were of approximately the same size collected from the same site, treated identically, and exposed to radioactive sea water simultaneously. However, since a size range, although small, did exist, the calcium deposition for each period of exposure has been examined with respect to various parameters of shell size by plotting the amount deposited against length, width, area, weight, and average thickness (area/weight). The scatter of points in each case indicated the absence of significant correlation. It should be pointed out that inasmuch as individuals used were selected for similarity of size, the present findings in no way indicate an absence of correlation between rate of deposition and size for a greater size range. Orton (1935) has shown a negative correlation between relative growth rate and size in the English oyster, while a low degree of correlation between rate of growth and size and weight of oysters has been reported by Havinga (1928).

The erosion of the inner surface which takes place when mollusks remain closed for extended periods suggested the possibility of removal of previously deposited calcium during the relatively long periods of closure which occurred in some individuals. Also, if deposition continued during closure, it might proceed at a different rate. To investigate the effect of closure, the time that each of the 60 oysters remained closed was determined from the kymograph records of shell movements. The deposition in individuals which remained closed for more than 25% of the experimental period appeared to be about the same as those which remained open for a longer time except for the 24-hour period where less deposition took place in the posterior region. (See Fig. 1.)

The observed variability in growth rate is not peculiar to the conditions of these experiments, for great variability is also the common finding in oysters growing in natural waters (Havinga, 1928; Loosanoff and Nomejko, 1949; Butler, 1952).

Discussion

The results presented indicate the feasibility of the use of Ca^{45} in studies of shell growth in the oyster. The use of radioisotopes offers two distinct advantages over conventional methods: (1) Extremely small growth increments such as those occurring in a few hours can be measured. (2) Differences in rate of deposition in localized areas can be determined. For comparative measurements of shell growth the radioactivity of a single area such as the posterior region can be used as an index of growth. Because of localized differences in deposition over the surface, total growth can be determined only by measurement of the radioactivity of the whole surface.

The isotope method for studying shell growth also imposes definite limitations. The experimental periods must be short in the case of rapid growth; otherwise absorption by layers overlying the material deposited previously will interfere with the measurement of the soft β radiation of the Ca^{45}. Because of the expense of isotopes and for practical considerations, the volumes of the radioactive solutions will normally be small, thus placing restrictions on the type of studies which can be carried out conveniently. Moreover, particular consideration must be given to the maintenance of a favorable environmental situation with small volumes. As judged by normal shell movements this requirement appeared to be reasonably well fulfilled in the present studies by placing a single oyster in one liter of sea water in a shallow dish and changing the sea water every four hours. Radiation injury is probably not a complicating factor inasmuch as the radioactivity of the medium can be kept relatively low. An obvious limitation of the method is that successive measurements of growth cannot be carried out on a single individual.

In addition to the active deposition of Ca^{45} by the oyster, a shell will become radioactive by an exchange of atoms of the shell for those in the surrounding fluid. As a result of this process of exchange a shell carefully cleaned of all tissue will take up increasing amounts of Ca^{45} as it remains in radioactive sea water. The extent of exchange in the shell of the living oyster cannot be estimated precisely without knowing the calcium concentration and specific activity of the fluid in contact with the inner shell surface; but on the assumption that these values are not very different from sea water (Hayes and Pelluet, 1947), an approximation can be made from the radioactivity of cleaned shells placed in the radioactive sea water with the experimental oysters. For the posterior region the exchange so measured was about 1% of the mean and 0.5% of the maximum amount deposited by the living oyster during 24 hours.[6] It is apparent that exchange offers no serious obstacle to the measurement of shell growth.

The non-uniform distribution of newly deposited calcium over the inner shell surface indicated differences in deposition rates which must eventually be correlated with cell structure and mechanisms in localized regions of the mantle. The low

[6] Exchange in shell will be considered in detail in a separate publication.

rate of deposition in the thin posterior shell margin is especially interesting. This finding does not in itself mean a lower growth rate but perhaps a high organic : inorganic ratio of material deposited. Orton (1928), working with English oysters, mentioned that this thin shell margin hardens later in the season; and Loosanoff and Nomejko (1949) found that the hardness and brittleness of the new growth of Long Island Sound oysters is greater as the environmental temperature increases. These findings strongly suggest that the type of material laid down by the mantle shifts with certain environmental changes. In view of this, the pattern of deposition found in this study might be quite different under other conditions. This could be studied by exposing oysters to Ca^{45} under the desired conditions and making an autoradiographic study of the inner shell surface.

Summary

1. The rate of shell formation in the oyster *Crassostrea virginica* Gmelin has been determined by measurements of Ca^{45} deposited by oysters placed in sea water containing Ca^{45}. The experiments were carried out at Beaufort, N. C., during May and June at 21.2 to 25.4° C. Radioactivity measurements were made directly on the inner shell surface.

2. The mean amount of deposition was directly proportional to the time of exposure to Ca^{45} for 8, 16 and 24 hours. Growth increments occurring in four hours were measurable. Individual variation in the rate of deposition was marked.

3. The mean rate of deposition in the posterior region of the shell was approximately twice that in the center and anterior regions. Within a general region local areas may exhibit a still greater difference in rate. From the average radioactivity per unit area of shell surface the total amount of $CaCO_3$ deposited in a given period can be calculated.

4. Shell from which all tissue had been removed became increasingly radioactive in sea water containing Ca^{45} as a result of exchange. The magnitude of exchange was relatively very small as compared with active deposition by the oyster.

5. Advantages and limitations of the isotope method for measurements of growth are discussed.

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ABSTRACTS OF SEMINAR PAPERS

JULY 1, 1952

An enzymatic study of yolk-platelet lysis. PAUL R. GROSS.

By means of a photometric method, the lysis of yolk-platelets isolated from amphibian eggs can be followed quantitatively. In this manner, a study was made of the rapid yolk-platelet dissolution which takes place in the presence of divalent cations, particularly calcium.

The lytic reaction was found to be pH dependent, with a sharp optimum near pH 6.0. When temperatures were increased beyond 60° C., the velocity of the reaction fell rapidly, although at 60–70° C., there was no microscopically observable damage to the platelets.

From yolk-platelet suspensions, it was possible to prepare a factor which then induced a more rapid lysis of other suspensions when Ca ions were added. Controls, to which this factor, previously boiled, was added, showed only the minimum reaction velocity for the system. Treatment of the factor with protein precipitants also destroyed its activity. The variation of lytic rate with concentration of this factor was, under certain conditions, linear.

Yolk-platelets may be lysed by hypertonic ($> 0.3\ M$) NaCl or KCl. If this lysate is dialyzed or diluted, there is a precipitation of an amorphous, lipid-containing protein material. This solubility change with respect to Na or K concentration is reversible. When, however, very small amounts of Ca (*e.g.*, $5 \times 10^{-6}\ M$) are added to the precipitate, this is rapidly and *irreversibly* solublized. The Ca-mediated reaction apparently alters the yolk material chemically.

The results of these and other experiments are interpreted as indicating the presence in yolk of an enzyme which is able to lyse the platelet in the presence of small amounts of Ca, Mg or Sr ions. This enzyme is not the phosphoprotein phosphatase first described in yolk by Harris.

The action of heparin preparations on cells and tissues.[1] ALFRED B. CHAET.

Heilbrunn has shown that substances which prevent blood clotting, such as heparin, also tend to prevent protoplasmic clotting. To study further the effects of commercial heparin on living cells, it was felt advisable to utilize the frog heart as a test object. In this study kymograph recordings were made of isolated hearts which were perfused with various concentrations of heparin (Hynson, Wescott & Dunning, Lederle, and Upjohn) dissolved in frog Ringer. When concentrations of heparin of 0.4–0.5% were used, a cessation of heart beat was observed. The heart remained in a relaxed state, as might be expected; and the effect was reversible. This effect is due to a substance capable of passing through a dialysis membrane, and therefore with a lower molecular weight than that of ordinary heparin. This relatively small molecule, obtained by dialyzing some commercial heparin preparations, is also capable of inhibiting cell division of fertilized eggs of the clam, *Spisula solidissima*, and of the starfish, *Asterias forbesi*. The inhibitory action of this molecule is lost with time when the preparation is stored at + 6° C., but retains activity for at least 8 days if frozen at − 22° C. The dialyzable fraction becomes metachromatic after remaining in contact with toluidine blue for several hours (heparin produces metachromasia immediately in the presence of toluidine blue). The dialyzed fraction has no effect on the clotting of blood when used immediately after dialysis. However, preliminary experiments indicate that under certain conditions the dialyzable fraction does have an effect.

The relatively small molecule present in some commercial heparin preparations is not phenol and is probably not any one of the other substances used in heparin purification. Indeed, there is evidence to indicate that the dialyzing substance may possibly be a breakdown product of the larger heparin molecule.

[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

An attempt to influence the survival time of cancerous mice.[1] L. V. HEILBRUNN, ATIDA HALABAN AND W. L. WILSON.

Experiments of Most in our laboratory have shown that when muscle is fatigued it gives off heparin or heparin-like substances (Most, in press, 1953). In view of the fact that heparin prevents cell division, and in view of the fact also that Bullough (1948) found that exercise markedly decreased the rate of mitosis in the skin of mice, we investigated the effect of exercise on the survival time of mice implanted with tumors. We used two types of malignant tumors, and the mice with these tumors were placed in cages containing an exercise wheel. The survival time of these mice was compared with the survival time of similar mice in cages without exercise wheels. Exercise appears to have a favorable effect on the survival time.

JULY 8, 1952

Studies on initiation of cleavage in the frog egg. J. R. SHAVER, S. SUBTELNY AND A. WANIA.

It has previously been shown that injection of centrifugal fractions of frog and mammal tissue homogenates into unfertilized frog eggs will produce parthenogenetic cleavage. A large part of the cleavage-initiating material is associated with particulate elements of the cytoplasm, especially larger granules. The capacity of cytoplasmic granules to initiate cleavage begins to appear in frog embryonic cells at late blastula, reaches a peak exceeding that of granules from adult tissue during a three- to four-hour period at early gastrulation, and decreases somewhat during subsequent developmental stages. Inhibition of development with sodium azide at gastrulation also inhibits the cleavage-initiating capacity of granules isolated from homogenates of such blocked embryos. Reversal of inhibition partially restores activity of the granules. Similar results have been obtained using p-chloromercuribenzoate, an agent known to inhibit the activity of sulfhydryl-containing compounds, the inhibitory effect of which can be reversed by treatment of granules with cysteine. Large cytoplasmic granules prepared from gastrula homogenates and subjected to ultraviolet radiation at 2537 Å lose their cleavage-initiating capacity after a dosage of 3960 ergs/mm.^{2} or more. Some preliminary results indicate the possibility of reactivating granules inhibited in this way with visible light. The results of these studies are interpreted provisionally as indicating: (a) the physiological role of the constituents of the cytoplasmic granules in establishing conditions favorable for initiation of cell-division; (b) the possibility that thromboplastic activity of granules may produce viscosity changes in egg cytoplasm necessary for spindle formation; (c) a quantitative and/or qualitative change in the constitution of cytoplasmic granules during development.

The structure of the "neuromotor apparatus" of Paramecium as revealed by the electron microscope. DOROTHY R. PITELKA AND CHARLES B. METZ.

The "neuromotor apparatus" of Paramecium, as seen with the light microscope, consists of a system of cilia, ciliary basal bodies (kinetosomes) and longitudinal, sub-pellicular fibers (kinetodesma) connecting adjacent basal bodies. In view of the presumed integrative function of these neuromotor fibers and the morphogenetic properties of the basal bodies, a more detailed study of this system seemed desirable. By appropriate technique (ultrasonic dissection of fixed animals) fragments of pellicle with associated basal bodies and neuromotór fibers were obtained for electron microscope study. The neuromotor apparatus of two species, *P. calkinsi* and *P. multimicronucleatum*, was examined. Starving, not dividing, animals were used throughout.

In *P. calkinsi* two basal bodies were associated with each pellicular polygon. Each "neuromotor fiber" consisted of not one fiber but a bundle (3–4) of fine parallel fibrils (approximate diameter 0.1 μ). Each fibril contained a spiral structure with a period of approximately 400 Å.

P. multimicronucleatum proved to be more favorable for a study of the relation of the neuromotor fibers to the basal bodies. In this form one basal body was associated with each pellicu-

[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

lar polygon. Again, the "neuromotor fibers" consisted of a bundle of several fine fibrils. Each of these fibrils arose independently from a ciliary basal body and joined the bundle. Each fibril extended for some distance as a member of the bundle and finally tapered to a fine point. Thus, the basic structure of the neuromotor system in *P. multimicronucleatum* is a bundle of fine fibrils. Each fibril arises from a basal body. These fibrils appear to be of constant length and overlap in shingle-like fashion along the bundle.

JULY 15, 1952

A visible response to stimulation in Spisula eggs. ROBERT DAY ALLEN.[1]

It has been observed that fertilization or artificial activation is followed by changes of shape in the eggs of the surf-clam, *Spisula solidissima*. Slight surface wrinkling results from application of agents which cause high percentages of germinal vesicle breakdown (activation). Some other parthenogenetic agents (heat, cold, urea, sodium and calcium ions) have little activating effect on *Spisula* eggs, but do cause deep surface indentations to appear. These structural changes cannot be induced in the absence of calcium. They are not osmotic effects, although they can be duplicated by removal of water with hypertonic solutions, or return to sea water from hypotonic solutions.

Haematocrit measurements clearly show that eggs with indentations have undergone a decrease in volume of at least 6-8%. This indicates that eggs lose water during their response to stimulation. Water loss (syneresis) in inanimate gels accompanies ageing or increase in rigidity. *Spisula* eggs which have aged 10-16 hours, or have been stimulated in the presence of calcium, show evidence of syneresis. An increased gelation can probably be inferred from the observed loss of water. Because of the brevity of the structural change, a relative viscosity increase could be detected only with prolonged indentations in eggs subjected to short heat treatment.

The frequency of indentations at the vegetal pole suggests contraction by the cytoplasmic gel, because the vegetal region of the egg contains the thickest band of cytoplasm. The properties of the cytoplasm are such that ultracentrifugation produces a ring of negative birefringence around the displaced nucleus. There is no apparent change in this birefringent region after fertilization.

The isolation and chemical characterization of the mitotic apparatus of dividing cells. DANIEL MAZIA AND KATSUMA DAN.

Methods have been developed for the isolation in quantity of the mitotic apparatus of dividing sea urchin eggs (*Strongylocentrotus purpuratus* and *S. franciscanus*). Essential features of the methods are (1) arrest of mitosis by means of 30 per cent ethanol at $-10°$ C., (2) stabilization of the MA by treatment with dilute H_2O_2, and (3) dispersal of the cytoplasm by dilute Duponol solutions.

The structure of the isolated MA at various stages of mitosis corresponds to the classical cytological descriptions. All of the major elements—nuclei or chromosomes, centrioles, spindles, asters and midbodies—are removed together as a single physical entity. The fibrous elements of the spindles and asters are positively birefringent with respect to their long axes as in the living condition.

A protein fraction has been separated from the isolated MA by solution in alkali and precipitation in the range pH 5-6. The protein accounts for most of the mass of the MA and for about two per cent of all the protein in the egg. Preliminary studies with the analytical ultracentrifuge reveal a single major component of particle weight *ca.* 45,000. There is evidence that the linking of the particles to form the fibrous structures of the MA involves S–S bonding.

Mitotic activity in relation to differentiation in the slime mold, Dictyostelium discoideum. J. T. BONNER. No abstract submitted.

[1] Public Health Service Fellow, National Cancer Institute.

Electrical method of "sexing" Arbacia and obtaining small quantities of eggs. ETHEL BROWNE HARVEY.

When you pass an electric current through an Arbacia, it sheds its eggs or sperm; a few eggs or a little sperm exude from each of the five gonopores, and the shedding stops immediately when the current is cut off. The procedure is as follows: Place the animal, dorsal (aboral) surface up, in a stender dish small enough to restrict it somewhat and just covered with sea water. *Alternating* current should pass from an ordinary wall socket through a Variac or rheostat to reduce the voltage to 10–15 volts. The electrodes (preferably platinum, as copper is toxic) are put at any two separate places on the shell. If copper electrodes are used, they should be covered with absorbent cotton, frequently changed. When the current is allowed to pass, within a minute the animal will shed; the eggs or sperm come from each gonopore in a fine line, the eggs tending to clump. The best eggs are those taken with a pipette immediately as they exude; these will give 100% fertilization and develop normally. By this method one may easily distinguish males from females by the color of the exuding sperm (white) or eggs (red), and "sex" them, separating males and females for further use. One can also obtain a few eggs at a time without sacrificing the animal. And one can use the same animal repeatedly to carry out experiments on successive days with uniform material.

The reason for the effect is that the walls of the ovary and testis contain smooth muscle cells which cause them to contract on electrical stimulation, thus forcing out the eggs or sperm.

A more elaborate electrical method was described two years ago by Iwata for Mytilus and a Japanese species of sea urchin, *Heliocidaris crassispina*. The simple method described above has been used successfully also for the American sand dollar, *Echinarachnius parma*, and can probably be used for many other animals.

Sperm of Arbacia will keep several days in a refrigerator at about 8° C. if kept concentrated in a small covered stender dish (about 3 cm. diameter). As is well known, sperm when diluted will last only a few hours. When a male is found by electrical stimulation it is best to cut it open and remove the testes as intact as possible for future use. By keeping sperm on hand and obtaining eggs by the electrical method, one can obtain fertilized eggs quickly at any time.

JULY 22, 1952

Further studies of the intercerebralis-cardiacum-allatum system of insects. BERTA SCHARRER.

Certain difficulties regarding the evaluation of the role of the corpus cardiacum in insects can be overcome by considering the organ not as a separate gland of internal secretion, but as a component of a neurosecretory system which is analogous to corresponding organ systems in crustaceans and in vertebrates. The concept that the corpus cardiacum serves as a reservoir for neurosecretory material produced by the pars intercerebralis of the brain and transported to the storage organ via the axons of the neurosecretory cells can be further substantiated by the effects of the interruption of the neurosecretory pathway connecting the brain with the corpora cardiaca. Severance of this pathway (nervi corporis cardiaci) in *Leucophaea maderae* leads to an accumulation of neurosecretory material proximal, and to its depletion distal to the site of operation. This result offers conclusive evidence that in the intact system the neurosecretory substance originating in the pars intercerebralis of the brain is transported via the nervi corporis cardiaci to the corpora cardiaca where it is stored. This interpretation of the morphological observations explains why no acute hormone deficiency seems to occur as a result of the extirpation of the corpora cardiaca.

The neurohypophysis of elasmobranch fishes. ERNST SCHARRER.

Recent investigations indicate that the hormones which can be extracted from the neural lobe of the pituitary gland, are not produced there (Bargmann and Scharrer, 1951). The neural lobe of higher vertebrates consists of glia cells, blood vessels, connective tissue, and nerve terminals. The latter store a protein substance which is elaborated by neurosecretory cells in the hypothalamus and which contains the active (oxytocic, vasopressor, antidiuretic) principles of the neurohypophysis. This concept is supported by observations in elasmobranch fishes in which the neural lobe of the pituitary is supposed to be absent. In fact, the essential components of the neural lobe, namely, the nerve terminals of the preoptico-hypophyseal tracts

are present, but they are distributed among the cells of the intermediate lobe. These terminals, like those in higher vertebrates, store the neurosecretory material produced by the cells of the preoptic nucleus. The sum total of these nerve endings should be considered as an equivalent of the neural lobe of vertebrates other than the elasmobranchs, the only difference being that in higher vertebrates the nerve terminals together with blood vessels and supporting tissue form a separate neural lobe.

Balantidial infection in tadpoles as recorded by cine-photomicrography. CARL CASKEY SPEIDEL.[1]

The ciliated protozoon Balantidium is the causative agent for the disease balantidiasis. In man this is accompanied by symptoms resembling those of amebic dysentery.

Balantidia may also cause balantidiasis in tadpoles. These parasites may invade the tissues either by way of the alimentary tube or by way of lesions in the skin. Motion pictures vividly reveal the various histological stages of such infections, both in tadpoles that eventually succumb and in those that recover. Pictures in natural color afford an effective demonstration of balantidia with ingested red blood cells in the circulating blood.

Among the scenes presented are the following: the invasion of the epidermis of the tail through experimentally induced wounds; the movements of balantidia through the subcutaneous tissues; the boring apparatus of the parasites in action; the rapid and conspicuous cytolytic effects caused by mass infections; the destruction and sloughing of epidermal cells; the formation of ulcers; the extravascular phagocytosis by balantidia of extruded erythrocytes in hemorrhagic zones; balantidia in the lymph and blood circulation; the intravascular phagocytosis of erythrocytes; the ingestion of various kinds of tissue fragments; and the multiplication of the parasites by binary fission. In tadpoles destined to recover, each invading balantidium is immobilized and destroyed by a group of leukocytes of the host.

JULY 29, 1952

Sperm agglutinins in the egg jelly of the frogs Rana pipiens Schreber and R. clamitans Latreille. G. S. BERNSTEIN.

Specific interacting substances (fertilizin and antifertilizin) have been obtained from the gametes of many invertebrates, but few vertebrates have been studied in this respect. The purpose of the present work was to determine whether sperm agglutinins were present in the egg jelly of several species of frogs. It was found that the egg water of *Rana clamitans* irreversibly agglutinates *R. clamitans* sperm and loses its agglutinating capacity after three or four days. Sperm agglutinins were not detected in egg water prepared from unovulated (jelly-less) eggs. *R. pipiens* egg water, however, did not agglutinate homologous sperm from September through the May breeding season. This may be due to the relative insolubility of *R. pipiens* egg jelly (the agglutinins are presumably located in the jelly layers), or *R. pipiens* fertilizin is possibly univalent. *R. pipiens* egg jelly is not visibly soluble in pond water, acidified pond water, or in low concentrations (10^{-1} to 10^{-6} *M*) of NaCl, urea or glycine. When placed in pond water, *R. clamitans* jelly swells to a greater extent than *R. pipiens* jelly. Both *R. pipiens* and *R. clamitans* jellies are soluble in alkali (5% KOH was found to be the best solvent), but the extracts were inactive.

Although these results show that sperm agglutinins are present in the jelly of *R. clamitans* eggs, the presence of agglutinins in *R. pipiens* egg water, as reported by Glaser in 1921, could not be demonstrated.

A function of the salivary glands of the larvae of Drosophila and other flies. G. FRAENKEL.[2]

The puparia of *Drosophila, Phormia regina* and many other flies are usually glued firmly to a surface. During the formation of the puparium, when the pupal contraction is almost finished, a liquid secretion flows out from the mouth and is, by movements of the anterior tip of

[1] Aided by a grant from the American Cancer Society (Committee on Growth).

[2] In collaboration with V. Brookes and H. Moorefield.

the body, distributed over the anterior section of the ventral surface. This secretion hardens within a few seconds and effects a firm attachment of the puparium to a substrate. The secretion is protein in nature. The development of the salivary glands has been described by Ross (1939) and Bodenstein (1943). The glands reach their largest size in the prepupa, after feeding has already ceased. Then they show high secretory activity with the product of this secretion finally collecting in the lumen of the gland. After the formation of the puparium all or much of this secretion has disappeared from the lumen. This secretion is of proteinous nature. The secretion in the glands and the glue by which the puparia are fixed to a surface have been studied by paper chromatography and appear to have a similar, if not identical, amino acid composition. Besides, both products contain free lysine and free glucosamine. The formation of this secretion takes place during the period when the salivary gland cells are largest and most favorable for a study of chromosome banding. This function of the salivary glands does not exclude the possibility that at an earlier stage, during feeding, they produce digestive enzymes. However, the puparia of many species of flies are never stuck to a surface and still show some secretory activity in the salivary glands of the prepupa.

Carotenoid formation by mutant strains of Neurospora crassa. FRANCIS HAXO.

Neurospora crassa normally synthesizes an elaborate mixture of C_{40}-polyenes comprising less than 0.1% of the dried mold. Some 14 components have been identified including acidic xanthophylls, neutral xanthophylls (spirilloxanthin, lycoxanthin, hydroxy-gamma-carotene), carotenes of different degrees of hydrogenation (lycopene, gamma-carotene, beta-carotene, delta-carotene, rhodopurpurin, neurosporene, and a "zeta-carotene-like fraction," actually a mixture of two new carotenes), and colorless C_{40}-polyenes (phytofluene and phytoene).

From among 8 mutant strains which showed an altered polyene composition, four types of mutants have been encountered: (1) specific pigment block, *e.g.*, elimination of the acidic fraction; (2) multiple pigment deficiencies characterized by elimination of xanthophylls and most carotenes, reduced synthesis of hydrogenated polyenes, and a normal phytoene content; (3) carotenoid-less mutants, apparently no carotenoid formed, normal or enhanced phytoene formation; (4) phytoene-less mutants, no phytoene detected, one or more pigments produced in greatly reduced amounts. Although at present the detailed findings cannot be reconciled in a simple biosynthetic scheme, possibly due to the complicating effects of modifier genes, they are consistent with proposals that acidic carotenoids represent terminal stages in carotenoid synthesis and that phytoene is closely related chemically to the well established C_{40}-polyenes. Furthermore, the finding of a phytoene-less mutant capable of forming even a small amount of carotenoid pigment suggests that colorless and colored C_{40}-polyenes may be formed by parallel lines of synthesis from a common precursor. So far, carotenoid deficiencies in Neurospora have not been correlated with any physiological abnormality. The phototropic response characteristic of the wild type strains is also displayed by albino mutants.

AUGUST 5, 1952

Meromyosins, the sub-units of myosin. ANDREW G. SZENT-GYORGYI. No abstract submitted.

Studies on the visco-elastic properties of the anterior byssal retractor muscle of Mytilus edulis. WILLIAM H. JOHNSON AND JOHN F. PERKINS, JR.

Inasmuch as the isometric myogram describes only rate of change of tension with time, additional information regarding the contractile system is obtained by a measurement of viscosity (mechanical resistance) and elasticity of contracting muscles. For example, the myogram does not tell whether the muscle is behaving like a linear spring which is stretched to a new length as a result of excitation (increase in tension without increase in elasticity) or whether such an imagined spring is not only stretched but also becomes stiffer (increase in elasticity). During the last two and one half years we have been perfecting a method for measuring the viscosity and elasticity of smooth muscles during contraction. This method is

described in another abstract in this issue of this journal as well as elsewhere (1950–1952). Results obtained to date indicate that there is a true increase in both the elasticity and the viscosity during isometric contraction produced by an electrical stimulus consisting of pulsating current. The elasticity increases from five to eight times and the increase in viscosity varies inversely though not linearly with the frequency of the applied mechanical sine wave.

Conduction in non-striated muscles. C. Ladd Prosser. No abstract submitted.

August 12, 1952

The separation and partial identification of the prosthetic groups of cytochrome oxidase and cytochrome b. Philip Person, W. W. Wainio and B. Eichel.

A method has been developed for the separation and partial identification of the prosthetic groups of cytochrome oxidase and cytochrome b. Na desoxycholate is added in graded amounts to insoluble heart muscle particles. The differential solubility of the components of the cytochrome system permits preparation of two fractions. One contains only cytochrome oxidase and traces of b and c; the other contains only b and c; no oxidase is present. Direct pyridine extraction of the first fraction yields a solution containing the reduced pyridine hemochrome of the cytochrome oxidase prosthetic group. From the second fraction, direct pyridine extraction removes only the prosthetic group of cytochrome b and not that of cytochrome c. The prosthetic group of cytochrome c, under these conditions, is so tightly bound to its protein that it will not complex with pyridine. The above extracts will remain stable for more than a month, without any further treatment, if kept in the cold. The absorption maxima of the reduced pyridine hemochrome of the cytochrome oxidase prosthetic group are at 430 and 587 mμ. Certain preparations show a slight additional absorption at 525–530 mμ. The absorption maxima of the pyridine hemochrome of the prosthetic group of cytochrome b are at 420, 525 and 555 mμ, in the reduced form. The absorption curve of the latter hemochrome is almost identical with that for the reduced pyridine hemochrome of pure ferroprotoporphyrin. Qualitative spectroscopic analysis, and quantitative chemical determination of the metals present in the cytochrome oxidase prosthetic group indicate that Fe is the metal component.

The oxidative pathway of carbohydrate metabolism in E. coli. Dwight B. McNair Scott.

By the oxidative pathway of carbohydrate metabolism, we mean the oxidation of glucose-6-phosphate to phosphogluconate by the enzyme glucose-6-phosphate dehydrogenase and the coenzyme, TPN, and the oxidative decarboxylation of phosphogluconate to pentosephosphate by phosphogluconate dehydrogenase and TPN. By the use of glucose-1-C^{14} this pathway has been shown to be operative *in vivo* in *E. coli* growing under aerobic conditions to the extent of about 35%.

Several of the enzymes of the pathway have been extracted from the bacteria and separated from each other. Optimal conditions for assay of the activities have been determined. Both enzymes were measured by the rate of reduction of TPN determined at 340 mμ on the Beckman spectrophotometer. When TPN and substrate were present in excess the rate was proportional to the amount of enzyme. Either Mg^{++} or Ca^{++} ions at a concentration of 0.015 or 0.02 *M* were necessary for optimal activity. The optimal temperature was 36° C. The optimal pH range of the two enzymes differed. Therefore by determining glucose-6-phosphate dehydrogenase at pH 8.5 and phosphogluconate dehydrogenase at pH 7.2, it was possible to determine the separate activities of the enzymes in extracts where both were present.

The enzymes extracted from *E. coli* could account for the oxidation of 4 to 6×10^7 molecules of glucose-6-phosphate and 3×10^7 molecules of phosphogluconate per bacterial cell per minute. During aerobic growth of *E. coli* at 36° C., glucose was used at the rate of 8×10^7 molecules per cell per minute.

Therefore, oxidative enzymes have been extracted to account for 37–80% of the total carbohydrate metabolized.

A comparative study of the ribonucleases of liver and kidney. JAY S. ROTH.

The characteristics of rat liver and kidney ribonucleases (RNases) in homogenates were determined and compared to crystalline pancreatic RNase. The latter is probably an extracellular enzyme with mainly digestive functions while the former are intracellular nucleases whose function is unknown.

Liver RNase had a pH optimum at 6.0 with a second smaller peak at pH 7.8, while the optimum pH of kidney RNase was 7.8 and it also had a second smaller peak at pH 5.9. This may indicate the existence of two enzymes in each tissue. Liver RNase activity in homogenates increased 20–30 per cent after 6–10 days storage at 0°, or after one day in a sample that had been frozen. Under these conditions there was no change in activity in the kidney enzyme. Mg^{++} inhibited the kidney enzyme almost 100 per cent, and the liver enzyme 33 per cent at 0.1 *M* concentration. The two enzymes responded differently to increasing ionic strength of Na^{+} and to heating.

From these and other properties it was concluded that the two enzymes differ from each other and from pancreatic RNase. The kidney enzyme is similar to pancreatic RNase in several respects, but liver RNase is quite different.

Addition of sulfhydryl reactants, p-chloromercuribenzoate, Ag^{+}, Pb^{++} and methyl glyoxal increased the activity of the liver enzyme in homogenates. Cu^{++}, however, was inhibitory to the extent of 50 per cent at 4×10^{-4} *M* concentration. The inhibition could be reversed with 2,3-dimercaptopropanol, but this reagent did not affect the reaction rate of the enzyme in control homogenates or those treated with the above mentioned sulfhydryl reactants. Evidence was obtained for the presence of an RNase inhibitor in liver homogenates which was largely or completely absent from kidney.

AUGUST 18, 1952

Cleavage synchrony in individual embryos and in populations of embryos of Arbacia punctulata. ALLAN SCOTT AND HOWARD FOX.

As successive tiers of cells develop within the embryo, a rhythm of division is established. The four vegetal cells of the 8-cell stage cleave from three to five minutes earlier than the four animal cells. The nuclei of the 16-cell stage divide, macromeres first (0 minutes), then mesomeres (+5 minutes), then micromeres (+10 minutes). The nuclei of the 32-cell stage are arranged in 5 rings, 8 animal-1, 8 animal-2, 8 vegetal-1, 4 vegetal-2 and 4 second micromeres. These rings divide in a progressive sequence: vegetal-1, 0 minutes; animal-2 and animal-1, 5–15 minutes; vegetal-2, 25 minutes; second micromeres undivided. The nuclei of the 64(60)-cell stage divide in a similar sequence (seventh cleavage) beginning with the vegetal-1b ring and progressing towards the animal pole, followed by the vegetal-2 ring of 8 cells, which divides so late as to occur simultaneously with the first divisions of the eighth cleavage, at which time the second micromeres are still undivided; temperature 24° C.

Populations of eggs fertilized simultaneously are well synchronized at the first cleavage but show a progressive loss of coordination in successive cleavages. Embryos fixed at five-minute intervals during the sixth cleavage, stained with Feulgen and mounted whole, show a gradual increase in the average number of nuclei per egg during 30 minutes. At 172 minutes of development 32.9 nuclei per cell, 177 minutes 33.8, 182 minutes 37.0, 187 minutes 39.0, 192 minutes 43.0, 197 minutes 50.8 and at 202 minutes 53.4 nuclei per egg; temperature 20° C.

Conclusions drawn from physiological measurements on individual embryos or on populations must be in accord with the rhythm of division in individual embryos and the progressive loss of synchrony in embryo populations.

The uptake of phosphate during the cleavage of the sand dollar egg. EDWARD CHAMBERS. No abstract submitted.

Oxygen consumption in single marine eggs with special reference to cell cleavage. P. F. SCHOLANDER, C. L. CLAFF AND S. L. SVEINSSON. No abstract submitted.

AUGUST 19, 1952

Comparison of dye elimination in the developing liver and kidney (pronephros). PHILIP B. ARMSTRONG.

When comparisons are made of dye elimination in the developing liver (*Ambystoma maculatum*) and kidney (pronephros of *Fundulus heteroclitus*), particularly as related to morphological and histological differentiation, striking differences are noted in the two organs.

Acid dyes forming true solutions were injected into both *Ambystoma maculatum* and *Fundulus heteroclitus* embryos. Trypan blue was also used.

1. The liver while rudimentary accumulated dyes against a concentration gradient; the pronephros did not.

2. Vascular channels did not appear essential to the elimination of dyes by the liver; elimination took place into the pronephric tubules only after vascular channels developed closely associated with the tubules.

3. Trypan blue was eliminated through the liver after the development of the Kupffer cells; it was not eliminated through the pronephros nor were reticulo-endothelial cells present associated with the pronephros.

Radioiodine uptake in the chick embryo thyroid gland. SEYMOUR H. WOLLMAN AND EDGAR ZWILLING.

Radioiodine uptake by the thyroid gland was studied in twelve 9-day and twenty-three 7-day chick embryos. Forty microcuries of I^{131} were injected into the allantoic vein. After a time interval (0.5 to 4 hrs.) blood samples and thyroids were removed for assay. The carcass below the neck was used as a non-thyroid tissue control. In a few cases samples of fluid and solid yolk, as well as allantoic and amniotic fluids were assayed. Organic binding of I^{131} in some of the thyroids was determined by homogenization in water and protein precipitation. I^{131} was measured by beta or gamma counts.

The I^{131} concentration in the blood fell rapidly after injection and was rarely found to be higher than that expected from a uniform distribution of the dose in the egg. The 9-day thyroids (colloid formation not visible) were found to concentrate I^{131} (ca. 5000 × that of blood); most of this was precipitable. The content of I^{131} in these thyroids was independent of time after injection and of I^{131} in the blood. The 7-day glands concentrated I^{131} but almost none of it was precipitable. I^{131} concentration in thyroids and blood decreased with time after injection, but the I^{131} concentration in the thyroids was 400 to 700 × that of blood at all times. I^{131} in the carcass was ca. 0.7–0.9 × that in blood. The fluid yolk accumulated the greater proportion (55%) of the total dose within a relatively short (1.5 hrs.) time.

These results demonstrate that the chick embryo thyroid gland may concentrate iodide before it acquires its ability to incorporate iodide into organic binding.

The glycogen body as a derivative of the avian neural tube. RAY L. WATTERSON.

A structural feature peculiar to birds is the glycogen body, a massive glycogen depot between the diverging dorso-lateral funiculi at the level of the four main dorsal roots of the sciatic nerves, separating the two halves of the spinal cord except for the interconnecting ventral white commissure.

Two views of its embryological source have been proposed: (1) an extra-neural origin from pia mater, arachnoid, or both; (2) an intra-neural origin from the wall of the neural tube.

An extra-neural origin after 7½ days of incubation seems ruled out by observation of specimens stained with glycogen-specific stains, since thereafter the reactive cells lie entirely within the roof plate and below the meninges. After 9 days the glycogen body is a single, mid-dorsal structure; between 8⅛ and 7½ days there exist distinct paired primordia separated by a septum of ependymal cells. Fusion of paired primordia progresses dorso-ventrally between 8⅛ and 9 days as this septum narrows and degenerates in dorso-ventral sequence. The youngest stage at which paired primordia can be identified is 7½ days; they are widely sepa-

rated by a broad ependymal cone and are directly continuous to either side with the adjacent inner mantle of the neural tube wall. Appearances suggest strongly that they represent the dorso-median portions of the inner mantle, *i.e.*, that they are derivatives of the neural tube wall.

Each lateral half of roof plates from three-day neural tubes displaced laterad above skin and feather germs produces a separate glycogen body. Entire roof plates of three-day neural tubes isolated from rest of tube form single glycogen bodies normal in size, shape and glycogen content. Both results favor presence of stem cells within roof plate at three days since relationships of future meninges to roof plate would be highly distorted by operations. Thus an extra-neural origin after three days seems unlikely.

GENERAL SCIENTIFIC MEETINGS

AUGUST 26–27, 1952

PAPERS READ

A contribution toward a knowledge of dominance and subordinance in the smooth dogfish, Mustelus canis (Mitchill). W. C. ALLEE AND J. C. DICKINSON, JR.

Dominance-subordinance social patterns having been observed in all classes of living vertebrates except Amphibia, Chondrichthyes and Agnatha, coupled with the fact that such behavior patterns have been well demonstrated in the teleosts, stimulated investigation of this behavior in *Mustelus canis* (Mitchill), the smooth dogfish.

Observation of this species, in groups of 6–10 individuals, revealed certain definite but simple behavior patterns of dominance-subordinance. In 268 noted meetings of two animals, where avoidance was necessary to prevent collision, the smaller individual avoided the larger 251 times. The avoidance motions are smooth and graceful. Aggressive action was observed on only three occasions. No evidence of territoriality was noted.

The behavior pattern of avoidance may be interpreted as an elementary pattern from which more complex types have evolved if it be assumed that this trait is a heritable one in the vertebrate phylum.

It is suggested that an investigation of dominance-subordinance in the Agnatha will prove most interesting and profitable.

The behavior of Spisula eggs with respect to potassium ions. ROBERT DAY ALLEN.[1]

Isotonic potassium chloride has been employed as a stimulating (parthenogenetic) agent for several marine eggs. In various systems potassium can act either as a stimulant or as an anaesthetic, depending on concentration and other factors. Isotonic potassium chloride will not activate eggs of the surf-clam, *Spisula solidissima;* however, if the potassium ion concentration of sea water is approximately doubled, germinal vesicle breakdown (activation) takes place. As the potassium ion concentration is further raised, increased exposure is required until finally the eggs fail to respond. Activation does not depend primarily upon potassium, for potassium-free sea water is quite as successful as excess potassium in initiating development, and both agents require the presence of calcium ions. When potassium is present in its usual concentration in sea water, Spisula eggs are prevented from developing spontaneously when shed. It is also true, however, that calcium and magnesium ions and other factors also contribute to this natural block to development.

There is a fundamental difference between activation by lack and by excess of potassium; a brief exposure to potassium-free sea water followed by return to sea water will cause activation. However, removal to sea water within five minutes after activation by excess potassium causes reversal of the developmental processes already initiated. This indicates some kind of priming process, the nature of which is unknown.

[1] Public Health Service Fellow, National Cancer Institute.

The potassium ion concentration also exerts a marked influence on the threshold of ultraviolet light stimulation. Increase or decrease from the normal potassium level in sea water results in a lowering of threshold.

Facilitation of hydrogen ion passage across the chorion of Fundulus by the chlorides of calcium, potassium and sodium. PHILIP B. ARMSTRONG AND D. R. SHANKLIN.

In a series of papers (1915–17, 1922) Loeb described the killing of Fundulus embryos by acetic acid and the delay of this killing by the addition of chlorides of potassium, sodium or calcium; concluding that the presence of the salts antagonized the passage of acid through the chorion. These results were in distinct contrast with those which Loeb obtained with gelatin-coated collodion membranes. The addition of salts to acid increased the rate of penetration of acid through these membranes. He concluded that different mechanisms operated with these two membranes. Armstrong (1928), using de-chorionated embryos of the same age as controls, demonstrated that the major site of Loeb's "antagonism" was at the ectoderm rather than at the chorion. In order to measure more accurately the passage of hydrogen ion across the chorion, the rate of change of pH of the sub-chorionic fluid was determined. The embryos were first washed in distilled water to rid them, insofar as possible, of the sea water salts in the membrane and sub-chorionic fluid, and then in dilute buffer of pH 8.4 to restore the sub-chorionic pH to that of sea water. The embryos were then placed in *M*/500 acetic acid, alone or in combination with salts and the change in sub-chorionic pH was measured by injecting pH indicator dyes into the sub-chorionic fluid. It was found that the addition of salts in most cases increased the rate of passage of hydrogen ions across the membrane and that the relative effectiveness of the chlorides was Ca > K > Na. Apparently the chorion of Fundulus is qualitatively the same as Loeb's gelatin coated collodion membrane, the passage of acid across both membranes being facilitated by the addition of salts to the acid. Therefore, the salt-acid antagonism appears to be exclusively at the ectoderm.

Nucleoprotein complexes of sperm nuclei. JAY BARTON II. See abstract in "Reports on Lalor Fellowship Research."

Studies on the poly-D-glutamic acid of Bacillus subtilis NRRL-571.[1] LEON S. CIERESZKO AND CHARLES R. CRANE, JR.

Bacillus subtilis, strain NRRL-571, produces on certain culture media, such as Sauton's, a polypeptide in which the only amino acid present is D-glutamic acid. Poly-D-glutamic acid is produced on simple media containing any one of the following substances: glutamic acid, aspartic acid, alanine, proline, and acetic, pyruvic, citric, aconitic, alpha-ketoglutaric, succinic, fumaric and malic acids. All of these substances are either components of the tricarboxylic acid cycle, or may readily be converted to such. Many of the common organic acids do not serve as sources of the polypeptide. We feel that these results indicate the operation of a tricarboxylic acid cycle in *Bacillus subtilis*.

Poly-D-glutamic acid may be prepared from clarified seven-day cultures of the bacteria on Sauton's medium by the following procedure. The solution is passed through ion-exchange resins to remove ionic material. The peptide stays in solution, and is recovered as the insoluble copper salt by addition of copper sulfate. The copper salt is brought into solution by the use of a complexing agent, such as Versene, and copper ion and added Versene are removed by ion-exchange. The colorless polypeptide solution may be lyophilized to yield a dry product.

Titration of the polypeptide indicates an apparent pH value of 3.9 for its carboxyl groups. The majority of the free carboxyl groups must be gamma carboxyl groups. The polypeptide has a maximum specific rotation in acid solution, and a minimum value in alkaline solution. Sedimentation of the polypeptide preparation in the analytical ultracentrifuge shows it to be homogeneous. The degree of ionization of the polypeptide greatly affects the sedimentation

[1] Aided in part by a grant from the Frederick G. Cottrell Fund, Research Corporation.

rate. Thus at pH 2.1 the sedimentation rate is approximately 1.5×10^{-13} while at pH 4.1 the sedimentation rate is 1.05×10^{-13}. The sedimentation rate falls further as pH rises.

The oxygen consumption of squid nerve. C. M. CONNELLY. See abstract in "Reports on Lalor Fellowship Research."

Study of the course of formation of the mitotic apparatus in Arbacia and Mactra by isolation techniques. KATSUMA DAN,[1] SUSUMU ITO AND DANIEL MAZIA.

The mitotic apparatus of *Arbacia punctulata* and of *Mactra solidissima* has been isolated successfully by the method described by Mazia and Dan (1952). The following observations have been made. (1) The germinal vesicle nuclei of unfertilized *Mactra* eggs, the nuclei of unfertilized *Arbacia* eggs, and the pronuclei of both types of eggs after fertilization are isolated in morphologically normal form by the method. (2) Monastral figures may be isolated from *Arbacia* eggs shortly after fertilization. (3) In *Arbacia* eggs, distinct spindle fibers are seen to run from the centrioles to the nuclear surface previous to the breakdown of the nuclear membrane. (4) In *Mactra* eggs, we do not observe asters in association with the pronuclei until just at the time when fusion begins. When the pronuclei flatten against each other, two asters are observed. The line connecting their centers runs through the plane of pronuclear contact. (5) In isolated metaphase and early anaphase figures of *Arbacia* eggs, phase-contrast observation distinguishes continuous spindle fibers from pole to pole and fibers corresponding in length and position to chromosomal fibers. The latter had not been observed in *Strongylocentrotus* eggs. (6) In *Arbacia*, the centrioles are frequently seen to have divided at some time during anaphase. (7) In the *Mactra* egg, which cleaves into blastomeres of unequal size, there is a remarkable difference between the two asters. The aster at the pole where the CD cell is to form is much larger than that where the smaller AB cell will form. This relation between size of aster and size of daughter cell in unequal cytokinesis is exactly as predicted by Dan (1943) in his theory of mechanisms of cell division.

The mechanism of nuclear breakdown in the Chaetopterus egg.[2] LESTER GOLDSTEIN.[3]

Within 7 or 8 minutes after Chaetopterus eggs are shed into sea water the germinal vesicle nucleus breaks down—a necessary prelude to the subsequent maturation divisions of the egg. An attempt has been made to elucidate the physiological mechanisms of this breakdown.

The process evidently occurs in two steps. First, within two minutes after shedding there is a loss of rigidity in the protoplasmic cortex of the egg and this is apparently associated with a release of Ca from the cortex. Once Ca has been released and has performed its function the breakdown process can proceed in the absence of Ca, thus indicating that Ca has activated another factor, probably an enzyme system. (This two-step system may be compared to the two-step system involved in blood clotting, only the first step of which requires Ca.) The nucleus, or the nuclear membrane, is then acted directly upon by the Ca-activated component or system.

It was postulated that the factor activated by Ca might be a proteolytic enzyme. This hypothesis was based on a number of lines of evidence, foremost of which is the fact that crystalline trypsin will produce nuclear breakdown following the removal of Ca from the cell. Moreover, trypsin is believed to enter the cell (rather than to merely function at the surface), since the enzyme apparently acts on the eggs even in media of pH 3.5–4.0. At this pH trypsin is inactive, while the cytoplasm of the egg is not markedly affected. Accordingly, with the foregoing data in mind, extracts of Chaetopterus eggs were made and tested for proteolytic activity by the Anson method, utilizing a hemoglobin substrate at pH 7.5. Preliminary results of these studies indicate that there is present in the eggs a proteolytic enzyme which performs rather much as predicted.

[1] U. S. Public Health Service Fellow.

[2] This investigation was supported in part by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

[3] Predoctoral fellow of the National Cancer Institute, U. S. Public Health Service.

A study of colloidal changes in sea urchin egg homogenates. PAUL R. GROSS.[1]

Arbacia eggs, freed of jelly, and of their Ca by washings in citrate and Ca-Mg-free artificial sea water, are readily homogenized. When Ca ions are added to such homogenates, a pronounced coagulation reaction takes place, and dense, fibrous aggregates form on the walls of the glass container. This reaction does not occur in the absence of Ca.

The reaction may be followed quantitatively as a decrease in the solubility (in water, 0.5 *M* KCl, and 1 *M* KCl) of the proteins of the homogenate. These solubility changes may be related to the similar changes described by Mirsky for fertilized sea urchin eggs.

The velocity of the clotting reaction depends upon the dilution of the homogenate. The speed falls, and the amount of Ca required to produce a given coagulation in standard time increases markedly, as the homogenate of centrifugally packed eggs is diluted with isotonic KCl. Experiments upon the early stages of the reaction indicate that a brief, transient *increase* in solubility may precede the clotting.

By means of a simple gelatin-digestion method, it was possible to show that the addition of Ca to homogenates induces proteolytic activity which is absent in the Ca-free preparation. The water-soluble proteins of homogenates were precipitated with $(NH_4)_2SO_4$. The precipitate was redissolved and dialyzed. The colloidal material in the sac also became proteolytic in the presence of Ca. This method permitted a determination of the pH optimum of the reaction. The optimum is in the neighborhood of pH 6.6.

These experiments, together with consideration of the inhibitors of the reactions described above, lead to a tentative hypothesis concerning the course of the coagulation reaction. This includes attack by a Ca-activated protease of sensitive protein molecules, which then aggregate.

Electron microscopy of some marine egg surfaces. J. HILLIER, A. I. LANSING AND T. B. ROSENTHAL.

Unfertilized, fertilized, centrifuged and uranyl nitrate-treated eggs of Arbacia, Asterias and Spisula (Mactra) were fixed in buffered osmium, sectioned at approximately 0.2 μ and studied with an EMU type electron microscope.

The cell surface of the unfertilized Arbacia egg, rather than being smooth is thrown up into small, fairly regularly spaced papillae into which the cytoplasm extends. There is no apparent vitelline membrane. The papillae of the fertilized egg are taller than those of the unfertilized egg. The fertilization membrane is readily recognized as a sheet less than 0.03 μ thick apparently composed of a single layer of loosely packed particles.

The surface of the unfertilized Asterias egg is quite similar to that of Arbacia. The papillae are somewhat broader and a vitelline membrane closely adherent to the surface may be distinguished.

The surface of the Spisula egg is quite distinctive. It is roughly 1 μ thick and possesses three characteristic layers through which long papillae or pseudopods course. The papillae arise from the cell surface as single stalks which branch into an average of four in the second layer. The three layers are of graded density with the outermost being most dense. Sections perpendicular to the long axis of the papillae show that they have a regular hexagonal-net arrangement in the outer layer, a less regular but similar arrangement in the middle layer and, in the inner layer where they join into the stalks, no distinguishable pattern.

Effect of temperature on the birefringence of the mitotic spindle. SHINYA INOUÉ. See abstract in "Reports on Lalor Fellowship Research."

The frequency dependence of the mechanical properties of smooth muscles. WILLIAM H. JOHNSON, JOHN F. PERKINS, JR. AND ARNOLD M. KATZ.

The elasticity and the viscosity (mechanical resistance) of the anterior byssal retractor muscle of the mussel, *Mytilus edulis*, were measured by a method described briefly as follows:

[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

A sinusoidal stretch is applied to the muscle and the sinusoidal changes in tension passively developed by the muscle as the result of the stretch are measured. From the knowledge of the amplitude of this tension, the amplitude of the sinusoidal stretch and the phase angle between the two sine waves, the elasticity and the mechanical resistance (viscosity) can be calculated in a manner analogous to the measurement of capacitance and resistance using the impedance bridge. These quantities, the viscosity and elasticity, were measured at frequencies of sinusoidal stretch ranging from 0.1 c.p.s. to 6.7 c.p.s. both during rest and during isometric contraction. In recent experiments the physiological response and the length at which tension first appeared upon stretching the muscle remained constant in most cases. The results obtained further substantiate earlier findings that the viscosity is frequency-dependent whereas the elasticity is virtually independent of frequency. The viscosity increases with decreasing frequency to a greater extent during contraction than during rest. This suggests greater cross-bonding during contraction (following Eyring and Tobolsky).

Electron microscopy of some marine egg inclusions. A. I. LANSING, J. HILLIER AND T. B. ROSENTHAL.

Ultra-thin sections of buffered osmium fixed eggs of Arbacia, Asterias and Spisula (Mactra) were studied with the electron microscope.

The cortical granules of Mactra appear to be homogeneous spheres while the granules of the other two eggs studied seem to be composed of spheres of a matrix material with peripherally embedded inclusions. Upon fertilization in Mactra there is an increase in the number of cortical granules at the surface followed by a sharp decrease in their density. Upon fertilization the cortical granules of Arbacia rupture, discharging the contents of the peripheral inclusions, and leaving the remaining vacuole-like structure as an extension of the cell surface.

Unfertilized Arbacia eggs centrifuged at high speed (75,000 rpm for 20 seconds) contain two layers of what appear to be mitochondria, one in the centripetal lipid layer and the other just above the yolk granule layer. The clear middle zone contains numerous elements described by McCulloch as the coarse fibrous component. However, serial sections show that these are sheets rather than fibers and are composed of double membraned lens-like bodies separated by cross striations.

As with light microscopy, the resting nucleus contains little formed structure but during division, very dense chromosomes may be seen.

Desoxyribonucleic acid in Arbacia eggs determined by isotope dilution. A. MARSHAK AND C. MARSHAK.

Methods previously used for DNA determination in this and related materials can be shown to be inadequate. The isotope dilution method with C^{14}-thymine is free of these errors. C^{14}-thymine was isolated from *Thiobacillus thioparus* grown in a medium where the only carbon source was $C^{14}O_2$ and purified by column and paper chromatography. The final product was at least 98% pure by radioactive and spectrophotometric criteria. Eggs were treated to obtain a soluble fraction containing RNA nucleotides and an insoluble residue containing DNA. No thymine was found in the former and no uracil in the latter, indicating adequate separation of DNA and RNA. On the basis of uracil determinations there was found to be 24×10^{-4} μg RNA/egg. The DNA fraction was digested with $HClO_4$ and C^{14}-thymine added immediately afterward. After equilibration, thymine was isolated from the digest by paper chromatography and purified by four successive chromatographic procedures. The plating and counting procedures were found by preliminary trial with C^{14}-glycine to be reproducible within an error of 1%. From the specific activities of the added and recovered thymine it was calculated that there was 8.4×10^{-7} μg thymine or 8.1×10^{-6} μg DNA per egg. Direct analysis by paper chromatography gave 8.2×10^{-8} μg thymine or 7.9×10^{-7} μg DNA per sperm. As demonstrated by cytological procedures, this result must not be interpreted as meaning that there is 10 times as much DNA in the egg nucleus as in the sperm.

Desoxyribonucleic acid in the nuclei of Arbacia eggs. A. MARSHAK AND C. MARSHAK.

Suspensions of extruded eggs washed and extracted as previously described were stained by the Feulgen reaction. Live preparations were examined by phase contrast. In addition to ripe ova, some suspensions contained oocytes and small fragments of the ovary. Oogonia and oocytes were enveloped by numerous syncitial or nurse cells with strongly Feulgen-positive nuclei, some of which appeared to be embedded in the egg cytoplasm and were frequently pycnotic. The nuclei of young oocytes have chromatin as typical paired strands that are very delicate but are definitely stained red. On enlargement of the nucleus the capacity to stain is lost. Chromosomes of the polar body divisions are bright red. However, there is no color in the pronucleus of the mature egg even though the chromatin strands are coarser than those of the early meiotic stages which do stain. The size of the pronucleus is not significantly different from that of nuclei through the 16-cell embryo stage. There is a progressive increase in intensity of staining of resting nuclei of successive cleavage stages. The evidence indicates a loss of DNA by nuclei in the early meiotic stages, and its absence in the pronucleus. The capacity of the pronucleus for producing DNA is indicated in the polar body divisions. The large amount of DNA found by chemical analysis of egg suspensions cannot be assigned to either the nuclei or the cytoplasm of the egg unless the presence of nurse cells is eliminated. Since Arbacia may develop parthenogenetically, it follows that DNA must be a derived product and not a prime originator of the reactions occurring at specific chromosome loci that are considered to be essential gene functions.

The osmotic behavior and anatomy of the Fundulus chorion. D. R. SHANKLIN AND PHILIP B. ARMSTRONG.

In 1905 Loeb stated that Fundulus choria were impermeable to water, later modifying this to "practical impermeability" under physiologic conditions and in distilled water. In 1908 he explained the collapse and crenation of the eggs in sucrose solution by exosmosis of salts. Yagle (1930) discussed this problem of salt and water passage, but failed to demonstrate endosmosis of water. By this study, the permeability of choria to water in both directions was measured. Exosmosis was measured by a manometric device, and passage of water both ways was measured by a geometric consideration of the collapse of the chorion in sucrose solution, and its recovery in distilled water or sea water. A mathematical treatment voids the salt exosmosis explanation for this behavior. Sucrose itself will pass this membrane, for a collapsed shell left in sucrose will recover, thence the yolk sac crenates and the embryo dies. These choria are resilient and spring back into shape with much force and speed. It is this quality that yields a discrepancy in the permeability of the chorion to water, for the water passage of recovery is three times that of collapse. If the chorion had a number of pores of sufficient size, this resilient "sieve" would "move through the water," and "capture" water to restore its spheroid shape. Microscopic examination reveals: (1) a chorion externum; 7–8 μ thick; with 10–20,000 pores/mm.^{2}, of 1–1.5 μ diameter; cylindrical with flanging at the outer opening, circular to polygonal in profile; randomly distributed; (2) a chorion internum; 3–4 μ thick; composed of protein fibrils 1–1.4 μ diameter, arranged in a herringbone manner, as a meshwork. Electron microscopic examination reveals no finer structure to the external chorion, thus a homogeneous layer of matrix, and the evidence that some of the pores pass both layers. The internal chorion is the site of osmotic regulation, while the outer affords mechanical protection.

Glycolytic activity in the swim bladder gland. C. F. STRITTMATTER, E. G. BALL AND O. COOPER. See abstract in "Reports on Lalor Fellowship Research."

The relation between the excystation of an apostome ciliate (Gymnodinioides sp.) and the molting of its crustacean host (Uca pugnax). WILLIAM TRAGER.

Small oval cysts of a species of *Gymnodinioides* have been found on the gills of over 80% of the fiddler crabs examined. Excystation of the ciliates has been observed only in material

taken from crabs during the act of molting or just before molting. The observations show that excystation begins just before the crab begins to molt, and that the excysted forms make their way into the shed cuticle of the legs during the act of molting. When fragments of infected gill taken from crabs during molting were kept in a mixture of sea water and blood of the crab, fully engorged ciliates (trophonts) developed within about 9 hours. Encystation of the trophonts and the development of small daughter ciliates required a further two days. Cysts on the gills of crabs not near molting underwent no change when kept for one or two days in the milky-white blood of crabs about to molt.

PAPERS READ BY TITLE

On the presence of lactose in the hypobranchial gland of the snail, Busycon canaliculatum (L.). METRY BACILA AND R. R. RONKIN.[1]

This note reports the occurrence of lactose, possibly as lactosamine, in a marine mollusk. Lactose is commonly considered a biochemical attribute of mammals.

Fresh mucus was obtained by agitating the excised hypobranchial gland of *Busycon* in a small volume of distilled or sea water. The secreted mucus was separated from the gland, precipitated with two volumes of 95% ethanol and allowed to stand three hours at 5° C. The precipitate was separated by centrifuging and decanting, washed by centrifuging three times with 95% ethanol, once with 100% ethanol and once with ether, and dried. The dried material, redissolved in water, gave positive biuret (protein) and Molisch (carbohydrate) tests, but after precipitation with $Ba(OH)_2$ and $ZnSO_4$ the supernatant was negative to both tests.

The dried, alcoholic precipitate was hydrolyzed by refluxing 50 mg. with 5 ml. 0.12 N HCl for four hours, cooled and centrifuged. The supernatant was neutralized to pH 7 with $NaOCH_3$ or NaOH and centrifuged. The clear supernatant contained free reducing sugar (Tollens's ammoniacal $AgNO_3$, Somogyi's and Nelson's reagents). Treatment with phenylhydrazine gave the characteristic crystals of lactosazone only, or, with more intensive hydrolysis, lactosazone, glucosazone and galactosazone. Paper chromatograms (solvent, BuOH-HAc; developer, Partridge's aniline-phthalate or benzidine) gave the same results, with the possibility that the lactose may have been in the form of lactosamine.

The polysaccharide was separated from fresh mucus by stirring 90 minutes with $CHCl_3$, centrifuged and the aqueous phase, containing no reducing sugar, precipitated with ethanol. The washed precipitate was hydrolyzed (0.5 N H_2SO_4, 100° C., four hours), neutralized to pH 4.5 with $Ba(OH)_2$ and centrifuged. The supernatant, concentrated by evaporation and chromatographed (solvent, phenol or BuOH-HAc with NH_3 and KCN added to each; developer, acetyl acetone and *p*-dimethylaminobenzaldehyde). The results suggested the presence of amino sugar and free hexose. It is possible that the amino sugar is glucosamine and that the hexose is galactose. Lactose, possibly as lactosamine, is thus the principal, if not the sole hydrolytic product of the polysaccharide of this mucus.

An ecological study of coastal plants on Cape Cod. STEVE G. BOYCE.

Salt spray, droplets of sea water concentrated by evaporation and transported inland by coastal winds, has been shown to be a factor in the ecology of plants along the shores of Cape Cod. The highest intensities of salt occur with the prevailing southwesterly winds and with storm winds of the northeast; consequently the espalier shape of coastal plants is oriented in these respective directions. The difference in species and in physiognomy on the southwest and on the northeast coast is due to the difference in frequency and fetch of the winds on the respective coasts. Increasing length of fetch increases salt spray intensity due to increased wave activity, increased swash and consequently increased effervescence of droplets. Anatomical studies of leaves of plants exposed to different intensities of salt spray showed that chloride-induced

[1] Aided by a grant from the Research Committee of the Faculty of the University of Delaware, and by a contract between the Office of Naval Research, Department of the Navy, and the University of Delaware (160-015).

hypertrophy was present in all species (32) except grasses. The degree of hypertrophy attained before coagulation of the protoplasm was correlated with the degree of tolerance of the species to chloride salts.

Modification of the tidal rhythm of Uca by tidal difference and by illumination. FRANK A. BROWN, JR., MURIEL I. SANDEEN AND MILTON FINGERMAN.

The integumentary melanophores of the fiddler crab, *Uca pugnax*, exhibit both a diurnal and a tidal rhythm. To determine the influence of time of low tide at two separate locales upon the phases of the tidal rhythm, *Uca* were collected at Chapoquoit and Lagoon Pond and their tidal rhythms under constant laboratory conditions compared. The time of occurrence of the maxima and minima of the tidal rhythm of the Chapoquoit *Uca* averaged 4.3 hours earlier than for the Lagoon Pond animals. The actual tidal difference between these two regions is 4 hours. It is, therefore, evident that the phases of the tidal rhythm of *Uca* are determined by the time of low tide in the environment. To demonstrate whether the tidal and diurnal rhythms of *Uca* are independent or associated with one another in some fashion, *Uca* were collected at Chapoquoit and divided into two groups. Both lots were placed in darkness at 9 P.M. on the day of collection. One lot was illuminated from midnight until 6 A.M. for three nights to shift the diurnal rhythm backward approximately a quarter cycle; the other lot serving as a control. A comparison of the times of maximum and minimum augmentation of the degree of dispersion of the melanophores indicated that the diurnal rhythm had actually been shifted backward five hours and the tidal rhythm 4.2 hours. The difference between five and 4.2 was not considered significant because of the difficulty of determining exactly the times of the maxima and minima of the tidal rhythm. It is evident, therefore, that the tidal rhythm is in some manner superimposed upon and associated with the diurnal rhythm. The tidal rhythm is probably adaptive for each environment since *Uca* habitually feeds at low tide.

A persistent tidal rhythm in the fiddler crab, Uca pugnax. FRANK A. BROWN, JR., H. MARGUERITE WEBB AND ROBERT C. GRAVES.

It is well known that the black pigment of *Uca pugnax* exhibits a diurnal rhythm of dispersion during the day and concentration at night and that this rhythm persists for several weeks in constant darkness. The state of the melanophores of a group of *Uca pugnax*, maintained in a photographic darkroom, was determined at hourly intervals from 5 A.M. to 10 P.M. inclusive, each day during a 26-day period. When these states are plotted against time in hours it is seen that the form of the daily excursion varies considerably from day to day. The curve may be almost perfectly symmetrical with maximal dispersion near noon; it may be skewed with maximal dispersion in either morning or afternoon, or it may be bimodal with a decreased dispersion at about noon. These periods of maximal dispersion were seen to bear a definite temporal relationship with low tides at the place the animals were collected. Further analysis of this relationship was performed by summing the chromatophore values for a given day and then determining the per cent of the total value represented by each of the six three-hour periods. The percentage values for any given three-hour period, when plotted against time in days, show a clearly rhythmical variation throughout the experimental period. The cycle so obtained has a frequency which approximates the 14.765 day half-lunar cycle of the tides. The minima (and maxima) progress from one 3-hour period to the next at the same rate as the tides. The observations indicate that the daytime dispersion of *Uca* black chromatophores is amplified in association with the time of low tide, that the precise time-relations between maximal dispersion and occurrence of low tide vary systematically throughout the day, and that this tidal rhythm persists for at least 26 days after the animals have been removed to a constant environment.

On the relationship between cleavage block by respiratory inhibitors and viscosity changes in Ilyanassa eggs. JOSEPH M. BUTROS.

To investigate the possibility that a change in the physical state of the cytoplasm (viscosity) links the chemical (oxidative) inhibition with the biological (mitotic) block, the following

inhibitory agents and anesthetics were used: *M*/200 sodium azide at pH 6.4; *M*/200 iodoacetic acid at pH 6.2; *M*/1000 KCN; 0.003% trichlorophenol; 0.01% dinitrophenol; 0.0018% maleic acid at pH 7; 1.5% thiourea; 0.06% phenylurethane; and 0.15% chloral hydrate. Viscosity of the eggs was studied by the centrifuge method; surface rigidity by the amount of elongation after centrifuging also. Some agents increased the viscosity, others decreased it; and still others did not affect it. In lower concentrations of some agents (azide) the delayed eggs cleaved in spite of the changed viscosity. Thiourea and chloral hydrate decrease the viscosity, maleic acid has a weak increasing effect. In azide, eggs became flattened, failed to cleave and became very viscous. When returned to sea water, they rounded up and after the return of viscosity to normal, they divided. Iodoacetic acid effect is like azide in general but irreversible after 20 minutes. Cyanide does not block cleavage, nor change viscosity, but causes the cortex to become more rigid. The substituted phenols increase the viscosity and make the cortex more rigid. In urethane the cortex is less rigid but the viscosity is increased.

From the above data, action of the agents on viscosity through cytochrome oxidase is eliminated. Other enzymes like phosphorylases could be involved. It is possible that these agents act directly on the surface and cytoplasm, changing viscosity and rigidity, respectively. It can at least be said that whenever a metabolic inhibitor blocked cleavage, it produced a change in viscosity and usually a change in surface rigidity.

Chemical characteristics of rotenone antagonists as determined by effects on dividing sea urchin eggs.[1] IVOR CORNMAN AND EDWARD F. ROGERS.

Using dividing sea urchin eggs, it was previously shown that vitamin E phosphate completely reversed a cytolysing dose of rotenone (0.008 mg./L) and that vitamin K_1 reversed weaker doses (0.004 mg./L). Related substances are being tested to discover the chemical characteristics necessary for rotenone antagonism. Vitamin K_1 oxide is partially effective against rotenone, but menadione (a mitotic poison itself, as shown by Heilbrunn and Wilson) and menadione phosphate are ineffective despite their vitamin K activity. Vitamin A has a carbon chain similar to that in K_1 and E. A-acetate and A-palmitate both partially reverse rotenone. Phytol alone is weakly effective in emulsions 10 times as dense as effective concentrations of the vitamins. It therefore appears that diverse ring structures (benzopyran, naphthoquinone, or partially saturated carbon ring) can improve the antagonistic action of the carbon chain even though a ring system alone (menadione phosphate) is ineffective.

In the range of 10 mg./L the vitamins in oil form neutralize the effect of rotenone to the amount of 0.001 mg./L. E phosphate, the most soluble form, requires 40 mg./L to neutralize 0.001 mg./L of rotenone. The fact that reversal of rotenone poisoning has been obtained only in the presence of precipitates or emulsions enjoins consideration of a purely physical action involving adsorption or solution, even though purely solvent action is inadequate to explain the difference between antagonistic and inactive oils.

On the other hand, the possibility of competition between vitamin E and rotenone at some site in or on the egg is strengthened by complete recovery of eggs blocked for an hour by rotenone (0.008 mg./L) before E phosphate is added. Washing such rotenone-poisoned eggs yields only abnormal blastulae.

Effects of P^{32} on mitosis in Chaetopterus eggs.[2] DONALD P. COSTELLO, CATHERINE HENLEY AND DONALD E. KENT.

Chaetopterus eggs were treated, beginning two minutes after insemination, by placing them in 90 ml. sea water containing varying amounts of P^{32}. The duration of exposure ranged from 40 minutes to 9 hours, and the concentration of P^{32} from zero to 0.07 millicuries per milliliter. The calculated total decay in the dishes containing the eggs ranged from zero to 100,000 R. E. P.,

[1] Aided in part by an American Cancer Society institutional grant to the George Washington University Cancer Clinic.

[2] Work done under A. E. C. Contract AT-(40-1)-1085.

though only a fraction of this dosage could reach the eggs. Egg samples were removed at intervals from the treated and control cultures, fixed as flattened whole mounts, and prepared for cytological examination by the Feulgen technique.

When a concentration of approximately 0.03 mc./ml. is reached, the early mitotic divisions are markedly affected. At this dosage, for eggs fixed after 40 minutes exposure, very large polar bodies, multiple asters in the cytoplasm and chromosomes of abnormal appearance are characteristic effects. Higher dosages (up to concentrations where all mitotic activity is stopped) result in chromosomal fragmentation and some multipolar spindles. Embryos fixed after three to five hours exposure show similar effects in the 8- to 16-cell stage cleavage blastomeres, plus some spectacular multipolar and polyploid figures. These latter often show large masses of chromosomes and apparent suppression of cytoplasmic divisions, reducing the cell number considerably below that of the controls. At the highest dosages used, both maturation divisions and cleavage were completely suppressed.

The fertilization inhibitor in the blood cells of Arbacia.[1] PIERRE COUILLARD.

In work frequently quoted, Lillie (1914) and later Lillie and Just (1924) stated that the perivisceral fluid of Arbacia inhibits fertilization. Oshima (1921) and more recently Pequegnat (1948) showed that the fluid itself contains no inhibitor and that Lillie's results were due to contamination by a "yellow substance" diffusing from pigmented haemocytes in the dermis during a preliminary washing of the animals with tap water.

As a matter of fact aged blood does inhibit fertilization. This is due to a substance, apparently echinochrome, which escapes from the pigmented blood cells.

Also Lillie's results might be explained by his use of filtered blood, for certain types of filter paper, in contact with blood or sea-water, yield a fertilization inhibitor.

Crystalline echinochrome was prepared by extracting blood cells and was found to inhibit fertilization. The effect is reversible; inhibited eggs fertilize readily if washed. If eggs are exposed after fertilization, echinochrome has no effect on cleavage. However, when fertilization is attempted in low concentrations of echinochrome, the eggs which are fertilized show on cleaving a separation of blastomeres such as occurs in calcium free sea-water. (A similar observation was made by Pequegnat.)

The binding of calcium by echinochrome was then investigated. Chemically, one mol of this compound could combine with 2½ mols of calcium (Tyler, 1939) and the complex formed precipitates in concentrated solutions. Addition of a calcium salt or of sea-water to echinochrome in distilled water modifies radically the absorption spectrum. Biologically the effects of echinochrome on fertilization and cleavage can be duplicated by solutions of potassium oxalate and prevented by excess calcium.

Mixed with sperm suspensions, dilute echinochrome causes a prolonged perhaps irreversible agglutination; in higher concentrations, sperm soon loses its motility, due presumably to a lack of free calcium (cf. Tyler, 1939).

It is suggested, therefore, that echinochrome acts primarily as a calcium binder in preventing fertilization.

The role of the midpiece in the incorporation of P^{32} into the ribonucleic acid of Arbacia spermatozoa. HENRY DI STEFANO AND DANIEL MAZIA.

Living Arbacia spermatozoa in sea water containing $P^{32}O_4$ (1 microcurie per ml.) incorporate P^{32} into the organic fractions insoluble in cold 10% trichloroacetic acid. The total activity incorporated into nucleic acids was estimated by measurements of the radioactivity of the fraction soluble in hot 5% TCA. In parallel samples, the partition of the P^{32} between DNA and RNA was determined by measurement of the activity released by crystalline desoxyribonuclease and ribonuclease, respectively. No detectable amount of P^{32} was incorporated into DNA, which is in agreement with observations on other types of cells. On the other hand, the amount of P^{32} liberated by ribonuclease was identical with that extracted with hot TCA.

[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

Therefore the sperm cell, in spite of its very small amount of cytoplasm and its lack of capacity for growth, does turn over or synthesize RNA.

A technique has been developed for isolating the midpieces of sea urchin spermatozoa in quantity. This involves, essentially, removal of tails by centrifugation in citrate solutions and heads by lysis in 2 *M* NaCl. When midpieces were isolated from samples of Arbacia spermatozoa that had been incubated in P^{32}, about 30% of the hot TCA-soluble P^{32} was recovered with the purified midpiece preparation. Since the method at present does not guarantee 100% recovery of midpieces, it is concluded that these particles are an important and perhaps the major center of RNA activity in the sea urchin sperm cell.

Comparison of the action of sodium adipate and sodium malonate on succinic dehydrogenase activity. BERTRAM EICHEL AND W. W. WAINIO.

Succinic dehydrogenase was assayed in rabbit liver homogenates spectrophotometrically. A concentration series of 8.3×10^{-6} *M* to 3.3×10^{-4} *M* sodium malonate in the presence of a fixed concentration of sodium succinate (3.3×10^{-3} *M*) gave 64 to 92% inhibition, while a concentration series of 3.3×10^{-6} *M* to 2.3×10^{-5} *M* sodium adipate gave 0 to 70% inhibition. A plot of 1/v vs. inhibitor concentration for these data (J. M. Reiner—private communication) indicated that normal inhibitor kinetics are applicable to this situation. The data also demonstrated that the amount of inhibitor-enzyme complex formed in each case as compared to the total concentration of inhibitor is not negligible.

It has been known that malonate behaves as a competitive inhibitor towards succinic dehydrogenase. The type of inhibition given by adipate, however, has thus far not been demonstrated.

With a fixed inhibitor concentration of adipate, 1.98×10^{-3} *M*, and malonate, 8.3×10^{-4} *M*, a concentration series of 8.3×10^{-6} *M* to 6.6×10^{-3} *M* succinate was studied in the presence of enzyme. Analysis of the malonate data by the method of Lineweaver and Burk, and Reiner, demonstrated that malonate does behave as a normal competitive inhibitor towards succinic dehydrogenase for all concentrations of succinate assayed. The Lineweaver and Burk analysis of the adipate data indicated possible competitive inhibition of the enzyme for a concentration range of succinate from 8.3×10^{-5} *M* to 1.65×10^{-3} *M*. Analysis by the method of Reiner ruled out the possibility of pure non-competitive inhibition and demonstrated that adipate behaves either as a pure competitive type or a mixed type of inhibitor. If adipate is a mixed type, its ability to combine with the free enzyme (competitive) far exceeds that of its combination with the enzyme-substrate complex (non-competitive).

Respiration and enzyme studies on X-irradiated Tetrahymena geleii W. HERBERT J. EICHEL AND JAY S. ROTH.

Recently, a study of the effects of total body x-irradiation on a number of rat tissue enzymes was completed (in press). It was believed that a similar investigation on unicellular animals might be of some aid in explaining the high radio-resistance of these organisms. The effect of x-irradiation on the activities of ribonuclease and desoxyribonuclease and on respiration under various conditions was studied. In each experiment standardized 48-hour cultures of Tetrahymena were concentrated by centrifugation, washed with distilled water and diluted to a constant volume. In respiration studies samples were removed for controls or to be irradiated and were then added to Warburg flasks containing 1.0 ml. of 0.1 *M* phosphate buffer, pH 7.4. Ribonuclease and desoxyribonuclease were assayed by the turbidometric method of McCarty on homogenates prepared from whole cell concentrates. Dry weight determinations were made in triplicate at 80° on the whole cells or homogenates. The activities of the nucleases were not changed significantly in homogenates obtained from cells which were irradiated at 300,000 and 500,000 r. This is to be compared with wide fluctuations in activity observed previously in the case of rat kidney and liver ribonucleases in animals, exposed to 500 or 750 r. When the oxygen consumption of Tetrahymena exposed to 300,000 r was measured immediately after irradiation, the QO_2 of the irradiated organisms decreased about 30%. After 24 hours, however, the respiration of the x-irradiated cells increased significantly above the controls.

Irradiation of the cells in the presence of 8.3×10^{-3} *M* L-cysteine and DL-homocysteine afforded approximately 45% protection. L-methionine gave no protection. It was noted that L-cysteine (final concentration 3×10^{-3} *M*) stimulated the QO_2 of normal cells by 34% while equimolar quantities of glycine, L-alanine, L-methionine, DL-homocysteine and glutathione had no effect on endogenous respiration.

The occurrence of mating types in Tetrahymena. ALFRED M. ELLIOTT AND DAVID F. GRUCHY.

One hundred twenty-seven clones of *Tetrahymena* sp. (probably *pyriformis*) were established from 15 different fresh water habitats in the Woods Hole area. Of these, three mated with themselves (selfers), whereas all others failed to conjugate within the clone (non-selfers). By making numerous combinations of non-selfers, both from the same habitat and from different sources, two mating types were finally discovered. These came from the same pond and mated readily with all clones from that source except one. Conjugation was obtained in only two of the remaining 101 clones.

Employing antibiotics, the two mating types were established in axenic cultures. They grow abundantly in proteose-peptone or the synthetic medium which is satisfactory for *T. pyriformis* strain E. Their nutritional requirements examined so far resemble closely those of strain E.

The procedure employed routinely in obtaining conjugation is as follows: Organisms (bacterized or axenic) are washed once with distilled water by centrifugation. They are then mixed in depression slides in distilled water, after which the first pairs appear in approximately 4 hours. If the original mating types are mixed in equal numbers approximately 100% of the cells will conjugate within 12 hours. The complete process requires about 8 hours, after which they separate and begin dividing, following a 5–6 hour period of feeding. They conjugate readily at room temperatures (18–26° C.) and at a pH above 7.0.

Improved culture conditions for Tetrahymena. A. M. ELLIOTT, J. F. HOGG, J. V. SLATER AND C. WU.

Studies were made on the physical and chemical factors involved in the growth of *Tetrahymena pyriformis* E. Of the physical factors, temperature and the effect of surface area were studied. Two temperature peaks were found for maximal growth rate (24° and 28° C.). The best growth was obtained when the ratio of surface area to volume was highest.

Of the chemical factors, studies were made (1) to discover whether the amino acid composition could be modified and lowered in amount with a view toward supplying available nitrogen from other sources, and (2) to determine the necessity for the addition of certain cations to the basal medium. When all l-amino acids were in the same proportion as in the medium routinely used, it was found that each could be reduced by one-half, with some reduction in growth rate. When amino acid levels were adjusted to twice the base level, on the basis of direct utilization data, and asparagine added to increase the amount of nitrogen, better growth was obtained than in the controls. Non-essential amino acid mixtures increased the growth rate and maximal growth when added to media constructed on the basis of minimal growth requirement data. The addition of asparagine to six times this base level resulted in the best growth rates and the highest maximal growth. This spectrum is recommended as the most economical for general use.

Magnesium and potassium are essential for strain E. This ciliate also probably requires less than one microgram/ml. each of copper and iron. Best growth is obtained when salts of magnesium, potassium, copper, iron and zinc are added to the synthetic environment. Without extensive purification procedures, however, it is impossible to demonstrate definitive cation requirements for this animal.

The effects of hematoporphyrin and other substances upon x-ray sensitivity of Paramecium. FRANK H. J. FIGGE AND RALPH WICHTERMAN.[1]

Various substances have been found to alter radiation sensitivity in vertebrates. In such multicellular forms, it is impossible to determine whether the effect is one which involves direct action of radiation on cells or an indirect effect due to complex secondary reactions and interactions of groups of highly specialized cells. For instance, stress reactions augment the influence of total body radiation in mammals. The protective effect of sodium pentobarbital in irradiated mice is perhaps related to the inhibition of stress reactions. Paramecia are ideal for the analysis of some of these radiation problems.

Using methods and conditions described elsewhere in these pages, specimens of *Paramecium caudatum* were irradiated in control media and in media containing hematoporphyrin, sodium nitrite or sodium pentobarbital. Paramecia in control media require very high dosages of irradiation to produce lethal effects (LD 50, 24 hr., 350 kr). Paramecia irradiated in chemically non-toxic porphyrin concentrations of 1:10,000 to 1:40,000 caused 100 per cent mortality with 100 kr within 12 hours. It is known that hematoporphyrin sensitizes paramecia to visible and near ultraviolet light. The above and other experiments demonstrate in a conclusive way that porphyrin sensitizes paramecia to short wave-length radiations such as x-rays.

Preliminary experiments with sodium pentobarbital and sodium nitrite in the concentrations employed (1:1000–1:4000) indicate that these substances do not protect paramecia from lethal effects of x-radiation. The protective effect of sodium pentobarbital in mammals is, therefore, possibly related to its tendency to suppress stress reactions which involve the nervous and endocrine systems.

The passage of vital stain from host to ectoparasitic wasp (Habrobracon). DANIEL S. GROSCH.

The present note reports a feasible means of experimental introduction of chemical agents into Habrobracon larvae. An intended application is to the investigation of the effects of ingested radioactive isotopes (A.E.C. Contract No. AT-(40-1)-1314). In order to have visible evidence of the distribution of substance introduced, 1% solutions of a chemically representative series of stains, most of them termed "vital" by stain technologists, were considered in these explorations. Paralyzed (pre-stung) caterpillars of the normal host, Ephestia, were given posterior injections using needles fashioned from glass capillary tubing. Habrobracon eggs were placed on the injected hosts and incubated at 30° C. Daily observations were made of the parasites which developed from the eggs.

With the notable exception of neutral red and toluidine blue, coloration was restricted to the midgut of the parasite and egested with the meconium. When these two stains were employed the appropriate color appeared in the urate cells. This phenomenon developed particular prominence with neutral red where the color persisted into the adult. Furthermore, pink cocoons were spun by the tinted parasites. Non-toxic stains restricted to the gut contents included fast green, alizarin red S, vital red, trypan blue and cresyl blue. Relatively toxic stains which caused premature deterioration of the host and death of the parasite include safranin O, Janus green B, trypan red and hematoxylin. Experiments with methylene blue were unsatisfactory because of extensive decolorization by the host. Only about 3% of the larvae fed on colored areas and showed slight coloration of urate cells. Although those stains which reached the urate cells belong to the quinone-imine group, it should be noted that cresyl blue and safranin O are also included therein. Therefore the structural chemistry of the chromophore groups cannot be advanced in explanation of absorption of a stain. Perhaps relatively low molecular weight and simplicity of the dye molecule as a whole may be significant.

Depolarization and contraction in smooth muscle on rapid cooling. RITA GUTTMAN, ARNOLD M. KATZ AND JOHN F. PERKINS, JR.

The anterior byssal retractor muscle of *Mytilus edulis*, the fibers of which are parallel and run the full length of the muscle, was rapidly cooled from about 24° to 7° C. Resting potentials

[1] This work was supported by the Committee on Research, Temple University, the Office of Naval Research, Department of the Navy and the American Cancer Society (Maryland Division).

were recorded on a Grass ink-writing apparatus through a cross-coupled input D. C. amplifier, A. C. being eliminated by means of a Wagner ground. A kymograph was used to record virtually isometric contractions. The muscle was placed in a lucite chamber. One end of the muscle was maintained in 0.48 *M* KCl and the other end was subjected to experimental solutions with a paraffin oil compartment between. Rapid cooling of the muscle in sea water caused reversible partial depolarization, but no contraction. Room temperature solutions containing three to five times the amount of KCl normal in sea water resulted in much slower partial depolarization and very slight contraction. Upon rapid cooling, these high potassium solutions potentiated a contraction accompanied by increased depolarization. Thus, the effects of cold and high potassium may be additive. Adding saturated cocaine in sea water to the 0.48 *M* KCl compartment resulted in no change of potential. Adding it then to the experimental compartment provided a true zero potential for the preparation. It is hoped that these experiments may contribute to the understanding of the relation between depolarization and contraction in muscle.

A further study of the mitotic inhibitor in starfish extracts.[1] L. V. Heilbrunn, A. B. Chaet, Arnold Dunn and W. L. Wilson.

Heilbrunn, Wilson and Harding (1951) showed that acid sea water extracts of starfish ovary have a marked liquefying and anti-mitotic action on Chaetopterus and Arbacia eggs. Such extracts also prevent cleavage of eggs of the clam Spisula and they prevent germinal vesicle breakdown and polar body formation in the starfish egg.

The potency of the extract is completely destroyed by periodate. This indicates that the effect may be due to a carbohydrate. If a carbohydrate is responsible, it appears to be combined with a protein. Autoclaving destroys the activity of the extract. When the crude extract is salted out with varying concentrations of ammonium sulfate, the potency remains in the globulin fraction. If preparations of crude extract are dialyzed in cellophane tubes, most of the active material remains in the dialysis sac, but enough active substance passes through the sac so that the dialysate prevents cleavage in Chaetopterus eggs. In general, therefore, we are led to conclude that the active principle of the starfish extract is a carbohydrate combined with protein. In view of the fact that the extracts are strongly metachromatic, the carbohydrate may well be an acid mucopolysaccharide of the heparin type.

K and Na content in dogfish leukocytes. H. G. Hempling.

Leukocytes of the smooth dogfish, *Mustelus canis*, were separated from the peripheral blood by mild centrifugation. The buffy layer was transferred to narrow tubes of 7 mm. diameter and 15 cm. length and mildly centrifuged again. The buffy coat was then re-suspended in either serum or elasmobranch Ringer's solution containing urea. The per cent distribution of cells obtained in this fashion were: small lymphocytes: 90.8; large lymphocytes: 3.7; thrombocytes: 2.8; eosinophils: 1.2; erythrocytes: 2.3. The cells were packed in the air turbine and, after obtaining wet weights, were cytolyzed in distilled water. Analyses were made on the supernatant, after removal of the residue, using a Beckman flame photometer. In 6 experiments, K values averaged 136.0 ± 13.8 meqs/kg. cell water. In 3 experiments, Na values averaged 88.7 ± 21.8 meqs/kg. cell water. Per cent water averaged 78.2 ± 1.0. Cells suspended in serum or in elasmobranch Ringer's containing urea, at 6° C. for seven hours showed no significant changes in total or differential count. K loss for this same period did not exceed 15% of the initial value. Cell water content remained constant. However, cells suspended in serum at 6° C. for two days showed a 92% decrease in total cell count.

The action of uranyl salts on the erythrocyte surface. Joseph F. Hoffman.

The effect of uranyl nitrate, acetate and chloride was studied on dogfish, tautog and human erythrocytes. The uranyl ion was found to be responsible since essentially the same result was obtained with each salt. Three types of phenomena were studied: (1) Clumping. Dilute suspensions of well-washed dogfish cells were clumped in concentrations of uranyl nitrate above 1×10^{-4} *m*/l.; the greater the concentration of uranium, the larger the average number

[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service, administered by L. V. Heilbrunn.

of cells per clump. Clumping could be almost completely reversed by reducing the uranium concentration by dilution or by the addition of phosphate. Clumping occurred at lower concentrations of uranium in tautog and human than in dogfish. The rate of clumping could be measured by the densimeter technique used in permeability measurements. (2) Shape. In nucleated erythrocytes uranium induced changes in cell shape ranging from crinkling to crenation; with human cells, from cupping and crenation to leaf shapes. These changes occurred in, and were dependent upon, uranium concentrations which did not cause clumping. (3) Permeability. The rate of exit of urea from dogfish cells was found to be reversibly inhibited in proportion to the uranium concentration. The maximum inhibition was ca. 200% that of the control limited by clumping at any higher concentration. The entrance of urea into the cells (slower than exit under identical gradients) could be inhibited, but to a lesser degree. Control of the pH or the presence of plasma protein had no appreciable effect on the rate or the per cent inhibition. Uranium had no measurable effect on the rate of passage of water, thiourea, glycerol or ethylene glycol. Glycerol entrance could be slowed in human and tautog erythrocytes, but not ethylene glycol or water. Since these results can be interpreted as surface phenomena, uranium is pictured as combining (reversibly) with the plasma membrane, presumably the protein components.

On the reproductive rate of two algal species after treatment with growth-affecting substances. JOHN W. KING.

Two algal plants, *Chlamydomonas media* and *Sphaerella lacustris*, were grown for 18 days on a medium of Knop's solution in 2 per cent agar with a trace of growth-affecting substance. Before the plates were inoculated, the solution containing the algal cells was drawn into a Thoma mixing pipette and shaken for three minutes. This was done in order that approximately the same number of cells would be contained in equal volumes of algal suspension. One drop of suspension from the mixing pipette was then placed on the experimental and control plates. The diameter of the circles made by these drops was found to be 6 mm.

After eighteen days in diffused light, considerable increases in diameter size of these growth circles were observed. The increases were as follows:

	C. media	*S. lacustris*
Control	.1 mm.	.1 mm.
Indole acetic acid	.4 mm.	.4 mm.
Indole-3-n-butyric acid	.4 mm.	.3 mm.
Indole-3-n-propionic acid	.2 mm.	.2 mm.
Colchicine	.6 mm.	.5 mm.

Microscopic examination of the cultures showed that the increase in diameter of algal circle was due to an increase in the number of cells. Therefore it was concluded that the number of algal cells varies directly with the size of the growth circle. It was also observed that *C. media* grown on agar culture containing indole acetic acid or colchicine was much greener in color and more luxuriant in growth than was the control.

Efforts are now being made to determine the per cent of increase during a particular period by making cell counts and by the spectrophotometric method. In order to determine the incessancy of this change in reproductive rate, transfers from experimental media to a number of control media will be made.

The influence of pituitary posterior lobe preparations on the action of phenolases in vitro. ROBERT R. KOHN.

It has been shown by Frieden and Bozer, and others that there is an increase in melanin pigment in vertebrates when a posterior pituitary extract rich in intermedin or when ACTH with intermedin activity is administered. Experiments with various hog posterior lobe preparations and phenolases were carried out colorimetrically in an attempt to extend or negate Fostvedt's unconfirmed results on the direct action of posterior pituitary fractions on mealworm tyrosinase.

Aqueous and dilute acid extracts, and re-suspended acetone and ether precipitates of posterior lobe extracts had no significant effect on mushroom tyrosinase-tyrosine reactions. Ex-

tracts boiled at a high pH were usually strong inhibitors of the reaction. An effective preparation was made according to the method of Landgrebe and Waring for intermedin, in which the oxytocic and pressor activities were destroyed, the active principle adsorbed on charcoal and eluted with acetic acid. This preparation shortened the lag period and removed the inhibition of mushroom tyrosinase caused by glutathione. Inhibitions of 28% and 31% were completely overcome while uninhibited reactions were stimulated up to 15%. Natural copper inhibitors may have been present in the enzyme preparation. Adequate controls were maintained.

The active preparation overcame glutathione inhibition of a *Palaemonetes vulgaris* catecholase-catechol reaction, but did not remove the lag period. Inhibitions of 33% to 48% were 75% to 83% removed, and uninhibited reactions were stimulated up to 36%.

A catecholase extracted from *Loligo* skin was inhibited up to 43% by glutathione. Inhibitions were completely overcome by the active pituitary preparation but the lag period was not consistently shortened. Uninhibited reactions were inhibited by the same concentrations of active preparation which protected against glutathione.

Effect and significance of various steroids and of ACTH (corticotropin) on the cleavage pattern of Arbacia ova. VALY MENKIN.

Cortisone acetate (Merck) (2.5 mg. in 10 cc. of sea water) tends to suppress the incidence of normal cleavage in fertilized *Arbacia punctulata* ova. This is not referrable to the vehicle in which the steroid is suspended. The vehicle does not alter the cleavage pattern. Filtering the suspension of cortisone acetate yields a cortisone residue capable of suppressing the incidence of normal cleavage, whereas the filtrate, which is essentially the suspending vehicle, has also no such inhibitory effect. The sperm are likewise inactivated by cortisone acetate (2.5 mg. in 10 cc. of sea water).

Desoxycorticosterone acetate and the glucoside tend to have the same inhibitory effect on the cleavage of ova.

ACTH (corticotropin) (Armour and Organon products) directly affects the cleavage pattern of Arbacia ova. There is also a greater incidence of suppression. This indicates that this pituitary hormone can likewise have a direct toxic effect on the ova without the mediation of the adrenal cortex.

In contrast, a protein anabolic steroid, testosterone proprionate (Ciba), has no effect on the incidence of normal cleavage in Arbacia ova. Somatrofin (STH, a Horner product), a growth hormone from the anterior pituitary, has likewise no effect on the cleavage pattern.

The suppressing action of ACTH is not a protein effect and it is not due to any differential solubility of the hormone.

The specific results suggest that the suppression of cellular activity, as measured by cell division, of cortisone and ACTH throws light on the mechanism of suppression of these hormones in inflammation as reported in 1940 and 1942 by the writer.

Activation of eggs of Nereis limbata by a surface active extract of dead sperm. W. J. V. OSTERHOUT.

If air was passed through the suspension of live sperm in sea water at room temperature little or no foam was produced. But if this suspension was heated for ten minutes at 55° C., thus killing all the sperm, a highly surface active substance was released. The entire extract was converted into foam when air was passed through it at room temperature. When this foam was dissolved in sea water and suitably diluted (pH value unaltered from that of the sea water) it activated the eggs. In favorable cases practically all the eggs extruded full jelly in a few minutes. Substantial numbers of them extruded polar bodies and if the eggs were transferred soon enough to a larger quantity of sea water, segmentation to two- and four-cell stages was observed. Later some abnormal trochophores were found.

Each extract was examined and found to contain no live sperm. The control was made by treating the sea water without sperm in exactly the same way. This had no effect on the eggs. Care was taken to avoid rise in temperature sufficient to activate. Experiments were carried out at 21° to 24° C. within three hours after collecting.

Every precaution was taken to prevent contamination by live sperm. In no instance did the experiment show contamination. Only normal eggs were used.

The same results were obtained with the extract which had been freed from the dead sperm by centrifugation.

Activating substance passed through a dialyzer but the inside solution remained more effective.

As mentioned last October in this journal a surface active substance in the sperm may play a role in activation.

Reversible contraction of protoplasmic structures by changes in pH values. W. J. V. OSTERHOUT.

The chloroplasts of the fresh water plant *Nitella flexilis* J. Ag. shrink in striking fashion when cells are killed in 0.1 *M* HCl, but regain their original size if well washed in distilled water. The entire process is reversible and can be repeated back and forth many times on the same chloroplast. Certain polyelectrolytes act in this way, presumably because negative charges on the molecule, which repel each other and make the molecule larger, are neutralized by the addition of hydrogen ions.

Further studies on the growth of oysters under different ecological conditions in Rand's Harbor. MADELENE E. PIERCE AND JOANNE E. CURRIER.

Studies on the growth of oysters begun in the summer of 1950 were continued in the summers of 1951 and 1952, during a 16-week period from June 19–Oct. 12. The same stations, representing four diverse locations, were maintained.

Bottom salinities at all stations remain very uniform, 29–32 p.p.m. Surface salinities vary widely, 1–32 p.p.m., and are often very low. A precisely defined film of cold fresh water, 5–6 inches deep, overlies the warm saline water. This film is particularly marked in the widened upper ends of the arms although it may extend along the margins toward the outlet to Buzzards Bay. The water which forms the film comes not only from the brook flowing into each arm, but also from the seepage of cold springs (12.5° C.) along the shore. Since this film moves up and down with the tide, the oysters living in the intertidal zone are bathed in it for several hours.

Temperatures, except in the film, are uniform on any one day, but fluctuate with the season. The pH is uniform for the saline waters, 7.8–8.1, but drops to 6.5–6.9 in the film.

A comparison of the plankton with the stomach contents of oysters showed a reasonable correlation. At the three stations near fresh water inflow the dominating forms were *Melosira, Pleurosigma* and *Nitzschia.* Near the entrance to Buzzards Bay, the dominating forms were *Chaetoceros,* copepods and veligers.

Except for the station in the stream, all trays of oysters showed about equal total growth in 1951 (results for 1952 are not yet complete). There was a tendency for more rapid increases in length and width to occur in the early summer, while that of depth occurred in the later part. Increase in volume occurred, however, at a uniform rate throughout the season. These results are in contrast to those of 1950 in which two trays outstripped all others in total growth of all dimensions, and all trays showed a significant increase in volume during late summer and early fall.

Fibrous ultrastructure in the hypobranchial mucus of Busycon. R. R. RONKIN.[1]

Observation of the remarkable flow properties of mucus prompted this search for their structural basis at the submicroscopic level. Smears of mucus from the hypobranchial gland of *Busycon canaliculatum* (L.), a prosobranch mollusk, were prepared on collodion films, dried in air, shadowed with chromium and photographed with the electron microscope at 7720 ×. Ropy strands pulled out of masses of fresh mucus were treated in the same manner. Dimen-

[1] Aided by the Research Committee of the Faculty of the University of Delaware and by a contract between the Office of Naval Research, Department of the Navy, and the University of Delaware (160-015).

sions in microns (means and their standard errors) are given for the characteristic structures observed.

Smears contained large elliptical bodies (length, $1.06 \pm .070$, $n = 5$; width, $.28 \pm .025$, $n = 6$), some with fine transverse striae (spacing, $.022 \pm .0015$, $n = 13$); an abundance of irregular, somewhat spherical bodies (diameter, $.074 \pm .0021$, $n = 50$) with irregular surface sculpturing, and masses of oriented fibers of indeterminate length (diameter, $.028 \pm .0026$, $n = 7$). Preparations of **strands** contained irregular bodies like those just described and broad, clearly defined ribbons of indeterminate length (diameter, $1.40 \pm .27$, $n = 6$). Margins of ribbons bore double edges, appearing as two opaque lines $.037 \pm .0038$ ($n = 10$) apart, separated by a clear space. Some ribbons appeared to contain longitudinally oriented fibers of about the same diameter ($.029 \pm .0025$, $n = 10$) as those found in the smears. The fibrous ultrastructure in both types of preparations may be significant in the interpretation of the mechanical properties of mucus and the birefringence of fresh mucus strands. No explanation is offered for the other structures observed.

Notes on the circulation in the yolk sac of the toad fish, Opsanus tau.[1] PAUL G. ROOFE.

The blood supply to the yolk sac is through the vitelline artery which is located in a more or less sagittal position posteriorly and ends frequently in bilateral branches which form a loop at the base of the yolk sac. The anterior convergence of this loop is the beginning of the vitelline vein which runs dorsally in an anterior sagittal position, ending in the sinus venosus. Between these two large vitelline vessels, numerous connecting links occur; some are true arteriovenous anastomoses; others are arterioles ending in capillary loops which converge to form venules which in turn enter the vitelline vein. The arterial twigs end in truncated cones as does the vitelline artery. The flow of blood is non-laminar, that is, it is turbulent. This turbulence has the appearance of a true "sludge," using Knisely's terminology. The basic units are two or three agglutinated red cells. The embryos are thrown into violent "spasms" of *S*-shape movements due to the accumulation of metabolic products in the internal environment. After each of such episodes, the turbulence of the blood is increased. Periods of inactivity allow the turbulence to clear up. The reason for the agglutination and its partial disappearance is unknown.

Responses of the distal retinal pigment of Crago septemspinosus to changes in illumination. MURIEL I. SANDEEN, MILTON FINGERMAN AND FRANK A. BROWN, JR.

The distal retinal pigment of the sand shrimp, *Crago septemspinosus*, shows active movements in response to changes in illumination. The normal time for maximal dark adaptation is about 90 minutes upon transfer from light to darkness and the time for complete light adaptation when animals are transferred from darkness to 250 ft. c. is about 30 minutes. The pigment also exhibits a smoothly graded relationship between the degree of its distal migration within the pigment cells and the logarithm of illumination over the range from about 0.00002 ft. c. to about 10 ft. c. Above 10 ft. c. no further light-adaptational response of the pigment is evident. There is also a distal pigment response to the shade of background when incident illumination is constant. The pigment is in a somewhat more dark-adapted position when the animal is upon a black background than when upon a white one. The response to background is not an albedo one, but is entirely explicable in terms of the influence of the background in determining the amount of light reaching the great majority of the ommatidia of the eye. Dark-adapted *Crago* respond to a one-minute, 250 ft. c. flash of light by an extensive but transitory light-adaptational response of the distal pigment, which reaches the fully light-adapted state in about 30 minutes, remains there for about another 30 minutes, and reaches the maximally dark-adapted state again only after four to five hours. This prolonged response to a brief light stimulus is interpreted as evidence for the abrupt discharge of a large amount of light-adapting hormone into the blood upon dark-to-light change.

[1] This work was aided by a grant from the Office of Naval Research, N6 ori T. O. 1.

The prevention of the iodoacetate effect on the sodium and potassium content of Ulva lactuca by photosynthesis. GEORGE T. SCOTT, HUGH R. HAYWARD AND WILLIAM D. ANDRUS.[1]

The influence of the glycolytic inhibitor, monoiodoacetic acid, on the sodium and potassium balance in living cells has been investigated by Wildbrandt (1940) on erythrocytes, Dean (1940) on muscle, Dixon (1949) on brain cortex and Scott (1951) on baker's yeast. An interpretation of the action of this agent can be based on its inhibitory effect on carbohydrate metabolism which is associated with ion transport.

The influence of monoiodoacetate on *Ulva lactuca* follows essentially a similar pattern resulting in a loss of K^+ and a gain of Na^+ under conditions of darkness. In the presence of sodium iodoacetate (0.001 *M*) for 24 hours the potassium content falls from 32 to 6 milleq./100 g. cell water, or to 20% of that of the controls. The Na^+ content increases from 11 to 30 milleq./100 g. cell water, which represents a 100% increase in "internal" Na^+. Time-course curves indicate a more rapid rate of ion transfer in material which had been kept in the dark for 24 hours prior to the experiment.

When the alga is maintained in the light during the course of the experiment, no loss of K^+ or gain of Na^+ compared to the controls occurs over a 24-hour period. Photosynthesis prevents the iodoacetate effect on these electrolytes.

A probable explanation of this observation is that the energy for ion transport and for the maintenance of normal Na^+ and K^+ balance is derived from the utilization of phosphoglyceric acid. Iodoacetate prevents the formation of this intermediate by its inhibition of phosphoglyceraldehyde dehydrogenase.

Under conditions of illumination photosynthesis provides the cell with phosphoglyceric acid which, according to Gaffron (1951), is the important intermediate in carbon dioxide reduction; hence, even in the presence of the inhibitor this material is available to cellular metabolism.

The rate and extent of exchange of K^{42} with cellular K^{39} in Ulva lactuca. GEORGE T. SCOTT, HUGH R. HAYWARD AND WILLIAM D. ANDRUS.[1]

Experimental evidence obtained principally on muscle and erythrocytes has shown that under physiological conditions a steady-state prevails in which the uptake and loss of K^+ are equal. By means of K^{42} it is possible to obtain information on this "equilibrium" state. The green alga, *Ulva lactuca*, offers favorable material for an extension of such investigations, since it consists of membranous fronds two cell layers in thickness, hence permitting a precise measurement of ion exchange between the cells and the surrounding sea water medium.

Small uniform discs of a frond were placed in sea water containing a tracer amount of K^{42} and maintained at three different temperatures in the light and in the dark. At various time intervals samples were removed, rinsed briefly in isotonic sucrose, weighed and counted for radioactivity. The material was then analyzed chemically for K^+ content by flame spectrophotometry so that specific activities could be calculated. The specific activity of the sea water was also determined.

These data indicate that the specific activity of the algae, $\frac{[K^{42}\text{ inside}]}{[K^{39}\text{ inside}]}$, reaches equality with the specific activity of the sea water medium, $\frac{[K^{42}\text{ outside}]}{[K^{39}\text{ outside}]}$; hence all the K^+ in the cell is exchangeable.

In the light at 10° C. 90% of the K^+ is exchanged in 4.4 hours; at 20° C., in 2.8 hours; and at 30° C., in 0.6 hour. Under conditions of darkness the rate of exchange is significantly less than in the light. Semi-log plots of the time course curves indicate essentially an exponential uptake of K^{42}.

The technique of photomicroscopy with the Polaroid Land camera. D. R. SHANKLIN.

When a concomitant record of the progress of an experiment is desired, conventional photomicroscopy being inadequate, the Polaroid Land camera is adaptable. The microscope used

[1] Aided by a contract between the Atomic Energy Commission and Oberlin College.

was an AO MH35 with oculars of various designs and manufacture giving 22.5 to 1405 diameters. A B&L student microscope was satisfactory, as was half of a binocular dissecting microscope. The technique follows: (1) the microscope is focused on the desired field, either vertically or horizontally; (2) condenser and diaphragm are set to desired light effect; (3) the light source is masked so that the only direct rays to reach the face of the camera do so through the microscope; (4) the camera is set on *bulb* exposure; (5) bellows are fully extended to the 3½ focus notch; (6) camera is adjusted so that the microscope centers a pencil of light through the diaphragm of the camera; (7) the pencil is focused by moving the camera to its smallest point on the surface of the shutter; (8) the smallest opening that will admit all of this pencil is set, usually No. 7 or 8, thus assuring sufficient light-tightness; (9) shutter lever is depressed for desired time; (10) activate prints for 60 seconds by standard Polaroid technique and observe results. Some times and situations that gave satisfactory results follow: 45×, 3 sec.; 97.0×, oil immersion, 15 sec.; 430×, dark field, 90 sec.; 1405×, wide field, 60 sec. Stronger light sources that gave readings on the light meter gave satisfactory results at the indicated *snapshot* shutter speeds. If one is willing to sacrifice one roll in adapting to his own microscopic equipment, he may find, as this worker has found, a valuable tool whose expense is palpable at reasonable application.

The effect of centrifugation and low temperature on development of Fundulus heteroclitus.[1] J. R. SHAVER AND SUSUMU ITO.

Previous studies have shown that centrifugation of eggs of *Fundulus heteroclitus* for 30 minutes at 300 g during the first 30 minutes after insemination produces a spectrum of abnormalities the most striking of which is duplication of parts or of the whole embryonic axis. The latter effect occurs in a small number of cases (ca. 1.0%) and it was thought that a combination of centrifugation and low temperature might increase the incidence of duplication, since the latter agent has been reported to produce this result in Fundulus. Eggs were centrifuged at room temperature over 0.83 *M* sucrose at 300 g for 30 minutes, beginning 10–22 minutes after insemination, placed immediately in sea-water at 4–6° C., and maintained at this temperature for 20–24 hours. Batches of eggs were concomitantly placed at room temperature, centrifuged and placed at room temperature, or subjected to the low temperature treatment. Mortality of embryos after the double treatment was 20% higher than that due to either agent alone, and the numbers of normal embryos were correspondingly lower. Numbers of abnormal embryos were not significantly different in the three experimentally treated lots. No duplications were obtained after exposure of eggs to low temperature alone. Twinning of parts or of the whole axis after centrifugation, or after centrifugation plus low temperature treatment, varied in different experiments, but the total percentages of these effects after the two types of treatment were not significantly different, and were of the same order as had been obtained by centrifugation alone (1.0–2.0%). A preliminary study of abnormal and duplicated embryos in section indicates deficiencies in embryonic organization and differentiation similar to those produced in amphibian embryos by centrifugation. The results of these studies are interpreted as indicative of an early formation of an organization center in the Fundulus egg comparable to that in amphibian eggs, the experimental alteration of which may result in some cases in splitting and axial duplication.

Survival of Spisula (Mactra) ova. VICTOR SCHECHTER.

Investigation of survival of unfertilized egg cells of the clam, Spisula, was made during the summer of 1952, in sea water of 23 to 24° C. In contrast to the results of experiments previously performed at temperatures of as much as seven degrees lower, it was generally found that cytoplasmic deterioration preceded germinal vesicle breakdown. Vitamin K (2-methyl-1,4-naphthoquinone), a coagulation factor, reduced survival time, even in concentrations as low as 1/50 milligram per 100 cc. of sea water. It was not found possible to stay this effect with either heparin or hirudin. Hydroquinone, in comparable concentrations, also shortened the life span, but the response of the egg differed in certain respects from that to vitamin K. Urea, known as a protein-lysin in higher concentrations, had a slightly beneficial effect in one per cent

[1] Aided by Summer Research Grant, University of Missouri.

solution. However, in combination with vitamin K, it increased the destructive effect of the latter. Dilution of the sea water medium produced various effects at different concentrations. The eggs swelled, demonstrating an intake of water. The egg membrane showed a capacity for stretching from approximately 7500 to more than 10,000 square microns before rupture occurred. In fresh water or in 10 per cent sea water bursting resulted in a few minutes; 40 per cent sea water caused rupture of the egg cortex and some loss of cytoplasm, followed by healing and fusion together of cells which happened to be in contact. At about 50 per cent concentration survival time was greater than in undiluted sea water, with an optimal point at approximately 70 per cent.

Effect of pH on "resting" Tetrahymena. JOHN V. SLATER.

Using a veronal-acetate buffer system it was found that "resting" cells of *Tetrahymena geleii* E. (approx. 2–4000/ml.) were able to tolerate a pH range of 6.4–9.9 for a period of 24 hours. When acid buffers were used (pH 3.7–5.8), the ciliates were killed but not cytolyzed. Values higher than 9.9 dissolved the cells. Stationary phase animals placed in buffers of pH 9.5 and higher (to 10.9) adjusted the pH downward. Animals killed in the acid range did not change the hydrogen ion concentration. Homogenates of stationary phase cells, of about the same number of animals as used in the tests, had no effect on buffers of high pH. Very heavy suspensions, however, when homogenized did lower the pH of buffers set at 9.6. The internal pH of this ciliate, as determined with homogenates, is 6.68. It is believed that living stationary phase cells, in the absence of the full nutrient spectrum, do appreciable work within a short period of time in adjusting the pH of their external environment.

Neural tube extirpation in Fundulus heteroclitus and resultant neural arch defects. RAY L. WATTERSON.

The role of the neural tube in neural arch formation is undergoing investigation in amphibians (Holtzer) and birds (Fowler and Watterson) by extirpation and transplantation experiments; this study extends the investigation to fish.

Attempts were made to extirpate a length of neural tube as cleanly as possible at Oppenheimer's stage 18 (4–14 somites); embryos were sacrificed after neural arch development was evident at unoperated levels. Operated specimens lacked spinal cord for a distance of 18–297 micra just posterior to the brain. In controls neural arches occur every 63–72 micra so longest extirpations covered a distance of at least four pairs of neural arches.

Results: (1) Typically no cartilage cells can be found lateral or dorsal to notochord at level of extirpation if nervous tissue is entirely lacking; (2) simultaneously with appearance of spinal cord anterior and posterior to level of extirpation cartilaginous neural arches appear; (3) if a small stretch of nervous tissue is present anywhere within level of extirpation, cartilage cells are usually associated with it; (4) badly disorganized nervous tissue can have relatively normal neural arches associated with it; (5) neural arches can in some instances be complete in dorso-ventral extent even though the dorso-ventral extent of the spinal cord is reduced appreciably; thus all dorsal skeletogenous cells which arise do not have to be in direct contact with nervous tissue.

Results are compatible with interpretation that the neural tube induces the somites to provide cartilage cells for neural arch formation and that the sclerotome does not arise by self-differentiation of the somite.

Occasional small clusters of cartilage cells lie dorso-lateral to the notochord in position of bases of neural arches in absence of spinal cord; these are usually closely related to adjacent haemal arches and presumably arise from them. Other clusters lie close to dorsal pigment cells.

The influence of light intensity and temperature on chromatophores of Palaemonetes vulgaris. H. MARGUERITE WEBB, FRANK A. BROWN, JR. AND ROBERT C. GRAVES.

The ability of *Palaemonetes* to respond to a white to black background change at light intensities ranging from 3800 to 0.00031 ft. c. was investigated by observing the response after

one hour. The maximum dispersion of the red pigment was obtained at 0.1 ft. c. with decreasing dispersion up to 3800 ft. c. at which intensity, however, the pigment was still somewhat dispersed. Between the light intensities of 0.1 and 0.00031 ft. c. there was an abrupt reduction in amount of dispersion until at 0.00031 ft. c. the red pigment was as concentrated as initially on the white background. The degree of dispersion of the white pigment on both the black and white backgrounds varied directly with the logarithm of the illumination from 0.1 to 3800 ft. c. Below 0.1 ft. c. the white pigment was maximally concentrated.

The chromatophore responses at a series of temperatures from 5° to 35° C. were also studied. The animals were permitted to adapt to the background for one hour and then to adjust to the temperatures for one hour. On a black background the degree of dispersion of the red pigment was greatest at 20° C. and became less as the temperature decreased to 5° C. and increased to 35° C. On a white background the pigment was maximally concentrated at 20° C. and 25° C. and tended to become more dispersed as the temperature decreased to 10° C. and increased to 35° C. At 5° C. the red pigment of the animals on the white background was found to be more concentrated than at 10° C. The white pigment behaved randomly with respect to temperature, with the pigment partially dispersed at all temperatures irrespective of background.

Environmental factors concerned with the lethality of x-rays in Paramecium. RALPH WICHTERMAN AND FRANK H. J. FIGGE.[1]

Specimens of *Paramecium caudatum*, cultivated on the bacterium *Aerobacter aerogenes*, were irradiated with two cross-fired x-ray tubes (180 kv. pk., 25 ma, no filter, intensity 7420 roentgens per minute). Temperature was determined during irradiation by means of a thermojunction placed in one of the control irradiation chambers. X-ray survival curves for microorganisms as reported in the literature vary, apparently depending upon the conditions employed for irradiation. We find, for instance, that under certain conditions the LD 50 may vary from 75 kiloroentgen to 350. The present work attempts to analyze the cause of variability and establish a standard, repeatable method. The ratio of the number of paramecia to volume of culture fluid is not nearly as important as the ratio of volume of air to the volume of culture fluid in plastic irradiation chambers of various sizes and shapes. Lethality of x-rays in Paramecium is related not only to the gradual accumulation of a toxic concentration of hydrogen peroxide and other similar compounds in the irradiated medium—a fact established long ago by many investigators using a variety of microorganisms—but our evidence indicates that it is also greatly influenced by a toxic substance coming from the air-space of the irradiation chamber. The rate of diffusion into the culture fluid depends on the surface area of the medium and its depth and volume. Attempts are being made to identify the toxic principle which may be derived from the oxygen in the air of the chamber, ozone or some other gaseous substance diffusing into the aqueous medium.

To eliminate factors which cause variation in survival curves, a 2-cc. nylon hypodermic syringe was used as an irradiation chamber. This device absorbs very little irradiation, eliminates air from the irradiation chamber and permits the introduction of various substances to be tested during irradiation. Accurate sampling of specimens after intervals of irradiation without changing the depth of the medium is also a desirable feature. It is thus ideal for the study of lethality of x-rays in Paramecium and may prove useful for similar studies with other microorganisms.

Effect of respiratory inhibitors on melanophoral activity of frog skin in vitro. PAUL A. WRIGHT.

In attempting to discover something of the nature of energy sources for melanophoral activity, a study was made first of oxygen consumption in excised pieces of frog skin (*Rana pipiens*) as detected in Warburg respirometers. In four tests, squares of blanched skin in Ringer's solution consumed 21.3 $mm.^3$ oxygen per $cm.^2$ per hour at 25° C., and no change in rate was observed when intermedin was added, even though darkening occurred as usual. In four subsequent

[1] This work was supported by the American Cancer Society (Maryland Div.), Comm. on Research, Temple Univ., and O.N.R., Dept. of the Navy.

tests, darkened skin in dilute pituitary-Ringer solution consumed oxygen at the same rate, which was again unchanged when blanched by addition of a concentrated adrenalin solution.

Using the photoelectric method for detection of color change in excised skin, a series of respiratory inhibitors was tested for possible activity in inhibiting either darkening or blanching. KCN (10^{-3} to 10^{-5} *M*), NaIAc (10^{-2} to 10^{-4} *M*), NaN_3 (10^{-2} to 10^{-4} *M*), urethane (10^{-1} to 10^{-3} *M*), or NaF (10^{-1} to 10^{-3} *M*), combined with 0.05 frog pituitary in 40 ml. Ringer's, had no effect on the darkening curves, nor was subsequent blanching in Ringer's solution delayed. Skins darkened by intermedin and then placed in the same concentrations of inhibitors blanched at the same rate as controls placed in Ringer's solution alone. Assuming that the inhibitors penetrated adequately, it appears that enzymatic energy-releasing reactions, which in other situations are inhibited by these chemicals, are not involved in melanophoral activity. These results bear a special significance in view of complete inhibition by all of these substances in appropriate concentration of ovulation *in vitro* in the frog recently reported by Rondell. Although the processes of ovulation and color change may not be considered wholly comparable, they are both pituitary-induced reactions, and the opposing results have been obtained under practically identical experimental conditions *in vitro*.

Effect of crustacean eyestalk hormone on melanophore activity in excised frog skin. PAUL A. WRIGHT AND ROBERT R. KOHN.

It has been reported by Abramowitz, Perkins and others that extracts of the eyestalks of several crustaceans are effective in darkening the skin of hypophysectomized frogs, and on the basis of these results and reciprocal data showing the appropriate response in Crustacea upon injection of vertebrate pituitary extract, an hypothesis has been proposed that the crustacean chromatophore hormone and vertebrate intermedin are structurally very similar if not, indeed, identical. Our experiments with excised frog skin (*Rana pipiens* and *R. clamitans*), using the photoelectric method for detection of color change, have led us to a wholly different conclusion. Of 46 tests in which extracts of eyestalks from *Callinectes, Palaemonetes* and *Uca* in dosages from the equivalent of 1 to 40 stalks in 40 ml. Ringer were used, one (10 *Palaemonetes* eyestalks) showed a response comparable to that produced by 0.05 frog pituitary in the same volume of fluid. This experiment could not be repeated, and the positive result may likely have been due to contamination of glassware with frog pituitary. Five other experiments using 6 and 10 *Uca*, 10 and 20 *Palaemonetes*, and two *Callinectes* eyestalks gave responses equivalent only to that produced by 0.002 frog pituitary in 40 ml. Ringer, and therefore can hardly be called conclusively positive. All other extracts elicited no darkening of frog skin whatsoever. Triturated eyestalks were extracted in Ringer's solution or in 0.7% NaCl, with and without boiling, and their activity for crustacean melanophores was ascertained by the fact that of several tested on eyestalk-less *Palaemonetes*, all gave positive results. Our data leave the impression, then, that the eyestalk hormone and intermedin are physiologically quite dissimilar, and raise the question whether positive results in hypophysectomized animals may not have been brought about through some other pathway than direct action of eyestalk hormone on amphibian melanophores.

Effect of colchicine on melanophoral activity in excised frog skin (Rana pipiens). PAUL A. WRIGHT AND JAMES SABAL.

The fact that colchicine arrests chromosomes in metaphase and also potentiates the effects of several classes of hormones, has prompted the investigation of its effect on melanophoral activity because this process involves, like chromosomes in mitosis, movement of a kind of cellular particulate, *i.e.*, melanin granules, and is under hormonal control by way of a secretion from the intermediate lobe of the pituitary gland. Exposure of excised blanched frog skin to 10^{-3} or 10^{-4} colchicine in Ringer's solution does not darken the skin nor affect in any way its ability to darken when immersed in intermedin solution, as determined by readings taken in a photoelectric colorimeter. However, subsequent blanching, which would normally follow upon transferring skin from intermedin solution to Ringer's solution, is almost totally inhibited, and melanophoral pigment remains permanently dispersed. Darkening occurs normally when skin is exposed to pituitary solution to which 10^{-3} or 10^{-4} colchicine has been added, but, again, blanching does not follow in Ringer's solution. If pituitary and colchicine are combined one

hour or more in advance of immersing the skin, partial (50–95%) inhibition of darkening may be observed and the melanophores are then refractory to intermedin alone.

Temporary blanching of a skin which has been darkened by a pituitary-colchicine mixture may be induced by adrenalin solutions, but melanophoral pigment disperses again after the adrenalin has washed out of the skin.

Colchicine is ineffective in preventing the blanching of a skin if it is applied after the skin has been darkened by intermedin alone. It appears, then, that colchicine enters into some sort of chemical combination with intermedin which prevents normal blanching of the skin. If colchicine and intermedin are allowed to react over a period of an hour or more, a compound may be produced which will partially inhibit darkening of the skin as well.

The bearing of new results in protein structure on cytological interpretations.[1] DOROTHY WRINCH.

Recent results suggest that the ribonuclease structure and the horse myoglobin and hemoglobin structures are made up of the same type of relatively small globulite molecules (20–25 Å across), in various numbers, various situated and various substituted (Wrinch, 1952). Following the general belief that globular proteins have a common type of structure, the cytoplasmic proteins, also, would be expected to comprise this same kind of molecule. In this event, it would be necessary to revise certain cytological interpretations. There are, in cytoplasm, systems of interlinked protein structures and other molecules which undoubtedly form continuous networks through various parts of or even through the whole of the cell. At present the protein fibrils therein are pictured as bundles of polypeptide chains, the protein in plasma membranes as such chains set in sheet-like arrangements. With the new type of structure for the proteins, *flexible three-dimensional networks* of proteins may be pictured (Wrinch, 1940). An immense variety of networks of globulite molecules, with various connexities and various water contents, can be worked out, capable of filling sphere-like or sheet-like or rod-like spaces. The difference between the two interpretations lies at the Å and $\mu\mu$ levels, the levels at which solutions of structural problems of cytology, such as the nature of cell permeability, have to be sought. The nature of the difference is best understood when we contrast a parallel array of polypeptide chains of atoms as the explanation of protein fibrils on the earlier view with the newer picture of such fibrils as chain-like arrays of essentially globulite protein molecules.

Recent results regarding the structures of the globular proteins.[1] DOROTHY WRINCH.

The new phase of the work on protein structure in the light of crystal data is based on x-ray studies of horse hemoglobin (Perutz, 1949) and myoglobin (Kendrew, 1950) and ribonuclease (Carlisle and Scouloudi, 1951). It consists in a comparative study of the intensity data and of the vector maps, maps concerned not with atomic positions but with the distribution of vectors between pairs of atoms in the crystal. Using results obtained in studies of the languages of intensity space (Wrinch, 1946) and vector space (Wrinch, 1939) we now have an apparently reasonable preliminary interpretation which provides definite indications regarding the type of molecules in the three structures. In each case the molecules are (1) cage-like in form, (2) globulite in shape and (3) relatively small in size, say 20–25 Å across, the skeletons of the molecules, presumably polymers of interlocked NCC backbones, being say 12–15 Å in diameter. From these dimensions, it follows that each structure contains a different number of molecules, perhaps 12 for the hemoglobin and three for the myoglobin and two for the ribonuclease structure. If these results are a guide to globular proteins in general, we would expect larger proteins to have more molecules and smaller proteins fewer molecules in their structures. In this event, sets of functionally related proteins in different species with the same MW may each be examples of some particular network, for example the hemoglobins of a 12-molecule network, and the myoglobins of a three-molecule, and possibly related, network. In any case, the variation in networks suggests a new aspect of the immense variety of the biologically active globular proteins.

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REPORTS ON LALOR FELLOWSHIP RESEARCH

Nucleoprotein complexes of sperm nuclei. JAY BARTON II.

Further study of the nucleic acid fraction of sperm nuclei resistant to removal by DNase has led to the following observations: (1) The fraction may be removed and recovered as a nucleoprotein complex by extraction of the DNase-digested nuclei with 2 *M* NaCl. (2) After removal of basic proteins by 0.2 N HCl, the resistant DNA can be easily removed by DNase.

Similar fractionation of the nucleus can be made by 2 *M* NaCl. Part of the nucleic acid of the sperm nuclei can be readily removed by cold NaCl. The remainder is removed only with difficulty if at all. The amounts of the removable fractions are as follows: Asterias, 72%; Mytilus, 73%; Mactra, 35%. These figures may be compared to the DNase-removable fractions: 85%, 84%, and 40%, respectively.

The DNA remaining after extraction with NaCl is readily removed by DNase. It is also removed by phosphate buffer alone. The phosphate extract is not a true solution but appears to be composed of fairly large particles dissociated from the remainder of the nucleus. The extract resists sedimentation at 19,000 RFC, but is brought down by 105,000 RFC. The DNA of the phosphate extract exists in a nucleoprotein complex very similar to that of the DNase-resistant complex in composition, though not in solubility.

These experiments distinguish between two of the nucleoprotein complexes of the nucleus: the first, widely studied, readily available complex of DNA and basic protein; the second, a physically separate fraction of DNA-protein characterized by its resistance to dissociation in strong salt solutions. The second fraction is not intrinsically resistant to DNase. Removal of the basic protein allows the enzyme to act. The resistance to removal by DNase can be interpreted as due to the spatial orientation of the DNA within the nucleus or to the unique mode of combination with proteins of the nucleus.

Studies on the distribution, potassium activation and mechanism of pyruvic phosphoferase. P. D. BOYER.

Pyruvic phosphoferase (which catalyzes the transfer of phosphate from 2-phosphopyruvate to ADP) from rabbit and rat muscle requires K^+ for activity. In the present studies, activation and distribution of this enzyme were measured by colorimetric determination of pyruvate appearance in a mixture containing enzyme, phosphopyruvate, ADP, Mg^{++}, K^+, purified yeast hexokinase and glucose, together with control measurements lacking ATP or K^+ or with Na^+ added in place of K^+. Pyruvic phosphoferase was found to be present and to show K^+ activation in all tissues examined including the following: muscle tissues from the marine forms skate, torpedo, Fundulus, Limulus, Pecten, Thyone and Phascolosoma; the fresh water forms Anodonta and Unio; rabbit heart, liver, kidney, uterus and brain; skate brain; and whole Tetrahymena. The enzyme from Anodonta and Unio showed considerably lower requirement for K^+ than the enzyme from marine forms or the rabbit. Kinetic studies showed that the inhibition of the enzyme activity by Na^+ and Li^+ did not conform to a simple competitive inhibition.

Measurement of exchange reactions between phosphopyruvate and pyruvate-2-C^{14} with a purified rabbit muscle preparation showed incorporation of radioactivity into the phosphopyruvate in the presence of enzyme, K^+, Mg^{++}, phosphopyruvate, pyruvate-2-C^{14} and ATP. The rate of reaction at equilibrium was only approximately $\frac{1}{1000}$ of the initial rate of phosphate transfer to ADP. The phosphorylation of pyruvate by ATP is likely the limiting step in the incorporation of pyruvate into muscle glycogen. Exchange was dependent upon the presence of ATP, thus ruling out the possibility that the reaction proceeds by formation of an enzyme-phosphate intermediate which subsequently reacts with ADP. The velocity of phosphate transfer to ADP is not decreased by ATP, which suggests that the combination of the enzyme with ATP is much weaker or independent of the combination with ADP.

Studies on the nonsaponifiable fraction of lipid material from marine animals. II. LEON S. CIERESZKO.

Cholesterol was isolated from the nonsaponifiable matter of the sand dollar, *Echinarachnius parma*. It was found that only traces of glyceryl ethers were present. Both these findings fit in with those of previous workers on the few other echinoids studied. The echinoids differ from the asteroids in that they contain cholesterol, while the latter possess stellasterols and the glyceryl ether, batyl alcohol, as major constituents of the nonsaponifiable matter.

Batyl alcohol was found to be absent from the skin fat of the common starfish, *Asterias forbesi*. The nonsaponifiable fraction of the skin fat contained about 10% of a substance which gave stable adducts with urea. This substance is presumably aliphatic.

The sterol of the colonial tunicate *Amaroucium constellatum* yields an acetate melting at 120–121°, and appears to be different from cholesterol. Cholesterol has been isolated from *Styela plicata*, the only tunicate previously examined for sterols. *Molgula manhattensis* also appears to contain a sterol not identical with cholesterol.

The oxygen consumption of squid nerve. C. M. CONNELLY.

A continuous flow respirometer utilizing the oxygen cathode has been employed to measure the oxygen consumption of stellar nerves of *Loligo pealii*, at rest and during activity, at 16° C. Giant axons of 400–450 μ diameter, stripped of accompanying small fibers of the stellar nerve, consume at rest 68 mm.^{3} of oxygen/gm.hr. (range 47–86, 8 nerves) (grams, wet weight). Whole stellar nerves, including giant and small fibers, have a resting consumption of 74 mm.^{3}/gm.hr. (range 62–95, 5 nerves).

Eight giant axons stimulated 50 or 100/sec. for 20 to 30 minutes showed during the first period of activity measurable increases to new levels of oxygen consumption, followed after activity by recovery to the previous resting level. Average increases were: for stripped giant axons conduction 50/sec., 3.7 mm.^{3}/gm.hr. (two experiments); conducting 100/sec., 4.2 mm.^{3}/gm.hr. (three experiments); for giant axons selectively stimulated in whole stellar nerve, the increases were 60% greater (three experiments). These axons stimulated for a second period or other axons stimulated for the first time showed much smaller or no measurable increase; in other nerves recoveries following increases were absent or incomplete.

Increases in the oxygen consumption associated with low-frequency activity in the small fibers of the stellar nerve are reproducible in the same nerve and are maximal in the range 29 to 43 mm.^{3}/gm.hr. (three nerves) at stimulation frequencies of 2 to 6/sec.

The increase in oxygen consumption per gram of nerve per impulse is thus many times (probably more than 50 ×) greater in the small fibers than in the giant axons. Studies of fiber size are being made to permit a quantitative test of the hypothesis that increase in oxygen consumption per gram per impulse is proportional to the surface area of the active fibers.

Comparative studies on ectodermal scleroproteins. MARCEL FLORKIN.

Electron microscopical studies pursued in collaboration with Dr. Ch. Grégoire have shown that the protein matrix of mother-of-pearl in molluscan shells is made of lace-like protein leaflets 2–4 mμ thick in gastropods and lamellibranchs and 4–12 mμ thick in the cephalopod *Nautilus*. Amino acid determinations, made in collaboration with Dr. G. Duchateau and Mr. H. Sarlet have shown that the protein concerned differs from the sclerotin of arthropod exoskeletons by a higher content of alanine and glycine and a smaller content of glutamic acid, histidine, isoleucine, lysine, proline, threonine, tyrosine and valine. On the other hand, while the water-soluble protein component of the lobster exoskeleton, arthropodin, shows the same composition as the scleroprotein of the same structure, sclerotin, the water-soluble component of nacre protein matrix shows a composition definitely different from that of the scleroprotein component of the same structure. These facts call for a comparative study of scleroproteins of ectodermal origin, in different animal groups. A number of materials collected in Woods Hole have proven suitable for such studies. Among them are the cuticles of *Mactra solidissima* *Busycon canaliculatum*, *Phascolosoma gouldii* and *Limulus*, and also the protein components of

the tubes of *Diopatra cuprea* and *Chaetopterus variopedatus*. Separation experiments have been performed on these materials and sufficient amounts have been collected for parallel electron microscopical and chemical studies.

Some aspects of blood coagulation in certain decapod crustacea. PAUL J. FREEMAN.

An attempt was made in this laboratory to choose from among representatives of the decapod crustacea varying type blood-clotting systems and determine whether the noted differences between them were qualitative or quantitative in nature. Also, comparisons were made between crustacean clotting and that of the better known vertebrate system. The methods used were as follows: 1. Factors known to be essential to vertebrate clotting were introduced separately into the non- or weakly clotting crustacean clotting system in an attempt to induce firm plasma clotting. A variation of this method was to use vertebrate blood with an essential clotting factor removed which was then added to crustacean blood. 2. Dilution of a firm clotting crustacean blood, *e.g., Callinectes sapidus*, with saline solution to a point of no clotting. This was then used as a control to compare dilutions made with non-clotting systems to determine whether clotting would occur at greater dilutions or not. 3. Use of anti-coagulants known to be specific in preventing essential vertebrate clotting factors from acting were tried on crustacean blood. 4. Comparative hematocrit determinations. 5. Observation of morphological changes in coagulocytes as clotting progressed.

The animals investigated may be divided into two major groups on the basis of their clotting reaction. Group I. No plasma clotting. This includes *Libinia, Carcinedes* and *Cancer*. Varying degrees of coagulocyte action were observed. Group II. Plasma clotting. This includes *Callinectes, Ovalipes, Homarus* and *Uca*. Coagulocyte action takes place first, followed by plasma coagulation.

The group I forms reveal no incipient plasma clotting factors such as coagulation enzymes or proteins. Hence, the differences in clotting reactions between the members of groups I and II appear to be qualitative in nature. *Callinectes* and *Ovalipes* reveal all the characteristics of vertebrate clotting. Sodium hydrosulphide prevents any coagulocyte action from occurring in the blood of members of both groups as revealed by absence of morphological cell changes and plasma clotting. This points to specialized blood cells as being the key factor in initiating all types of coagulation in decapod crustacea.

Effect of temperature on the birefringence of the mitotic spindle. SHINYA INOUÉ.

The birefringence of the metaphase spindle of the oöcyte of *Chaetopterus pergamentaceus* can be abolished when the temperature is either lowered below 4–5° C. or raised above 30–35° C. Upon returning the preparation to intermediate temperatures, a smaller spindle reappears, almost immediately, at the position where the original spindle had disappeared. At 25° C., the new spindle regains the full size in approximately 15 minutes. In eggs treated wih cold, the time required for the re-establishment of the spindle is not affected by the duration of the cold treatment, even up to several hours.

During the reconstitution, the spindle goes through a regular series of morphological changes. First the small spindle migrates rapidly to the cell periphery, then moves slowly away from the surface again as the retardation and length of the spindle increase. At this stage, continuous fibers show strong birefringence, but after the retardation has reached a maximum, chromosomal fibers replace the continuous fibers. The retardation of the individual chromosomal fibers fluctuates for some time until finally the chromosomes are regularly arranged on the metaphase plate. Once the spindle is completed, the birefringence of the spindle is fairly stable for a given temperature.

The equilibrium retardation is a function of temperature, and is greater the higher the temperature up to a maximum at 28–30° C. At the same time, the retardation of the spindle increases proportionately with spindle length. Since the spindle tends to be longer at higher, and shorter at lower temperature, it would appear as if the temperature dependence of the retardation were correlated with the change in length. However, it can be shown that even at equivalent lengths, the retardation of the same spindle is greater at a higher temperature.

Studies on the distribution of vitamin B_{12}. CHARLES A. NICHOL.

Vitamin B_{12} is required by many animal species; in man, lack of this vitamin causes pernicious anemia and in many mammalian and avian species an inadequate supply of vitamin B_{12} results in depression of growth. However, yeast and the tissues of higher plants are apparently devoid of vitamin B_{12}. Supply of this vitamin is dependent upon the synthetic ability of many microorganisms; other microorganisms requiring vitamin B_{12} are useful in sensitive assay procedures. In this study, measurement and identification of vitamin B_{12} were made by means of a microbiological assay (*Lactobacillus leichmannii* ATCC 7830) and paper chromatography.

The present investigation was directed along two lines: (1) *Distribution in tissues of invertebrates.* Our knowledge of the distribution of vitamin B_{12} in invertebrate species is very limited. Several reports have indicated the presence of vitamin B_{12} in certain molluscs and annelids but in each case, assay of the whole organism prevented relationship of the activity to the metabolic processes of particular tissues. In this study, extracts of tissues from representatives of invertebrate phyla were prepared for analysis. With few exceptions, clearly defined tissues were separated for treatment. (2) *Distribution in relation to function.* Vitamin B_{12} has been implicated broadly in the metabolism of nitrogen and in the formation of methyl groups. In the marine elasmobranchs a very large proportion of the waste nitrogen is detoxified and excreted as trimethylamine oxide. Since this product can be reduced to a volatile amine and readily isolated, study of the formation of trimethylamine oxide should be advantageous to the detailed study of the function of vitamin B_{12}. As a preliminary step in such a study, tissues of several elasmobranchs (smooth dogfish, ray and torpedo) were prepared for analysis for vitamin B_{12}.

Glycolytic activity in the swim bladder gland. C. F. STRITTMATTER, E. G. BALL AND O. COOPER.

The present study is a contribution toward clarifying the possible role of the so-called red gland of fish swim bladders in the selective release of oxygen into the swim bladder. It has been suggested that the oxygen is released from oxyhemoglobin of the blood as the result of the metabolic production of an acid by this gland.

The acid production of swim bladder glands from the common scup was followed *in vitro* by manometric determination of carbon dioxide release in a 0.225 *M* sodium chloride-0.025 *M* sodium bicarbonate medium at 30° C. with an atmosphere of 5% CO_2-95% N_2. Usually each gland was bisected and one-half run as a control. Addition of glucose (75 mg. % final concentration) increased acid production ten- to twenty-fold, to about 6 micromols per 100 mg. fresh gland per hour. Mannose, fructose and galactose in this same concentration produced 87.5, 30.5 and 14.2%, respectively, of the activity observed with glucose. The activity observed with glucose as substrate was inhibited about 75% on replacement of the sodium in the medium by potassium. Addition of phosphate or of magnesium or calcium ions to the limit of solubility in the medium produced no appreciable effect on acid production. Changing the pH of the medium from 7.3 to 7.0 caused a 50% drop in acid production; a rise to pH 7.6 produced a 25% increase.

The acidic product of glycolysis was identified as lactic acid. Under anaerobic conditions and with an initial added glucose concentration of 75 mg. %, lactic acid formation accounted, on the average, for 75.5% of the glucose utilized and 90.3% of the acid production. These percentages were somewhat lower when no glucose was added and slightly higher with 150 mg. % glucose. The same pattern of metabolism was observed under aerobic conditions (5% CO_2-95% O_2), both with respect to amount of glucose utilized and the percentages of utilization and acid production accounted for by lactic acid.